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Prostate Cancer

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13. ABSTRACT (Maximum 200 Words) <p>Prostate cancer is now the most common cancer among men in the United States. Angiogenesis is required for prostate tumor survival, growth, and metastasis. We proposed to design novel Hunter-Killer Peptides (HKPs), each representing a chimeric peptide of an angiogenesis-targeting peptide and a mitochondrial membrane-disrupting peptide. While non-toxic in the circulation, the HKPs will be preferentially toxic to mitochondria once internalized into angiogenic cells, via the targeting domain. As we reported in Ellerby et al., <i>Nature Medicine</i>, 5, 1032-1038, 1999, our prototypes contain only 21 and 26 amino acid residues, are selectively toxic to angiogenic endothelial cells and show strong anti-cancer activity in mice (breast carcinoma xenografts). In the work described here, we evaluated the HKPs for efficacy and toxicity in a xenograft model of human prostate carcinoma, and in the TRAMP (transgenic adenocarcinoma mouse prostate) model for prostate cancer. The central theme of this research is to develop and appraise this new chemotherapy with the goal of producing both a safer, and more effective, treatment of advanced prostate cancer.</p>			
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Introduction

The subject of the research is prostate cancer. The purpose of the research is the development of a more effective and less toxic prostate cancer treatment. A critical limitation of the currently used chemo-therapeutic agents is that these are the drugs with the narrowest therapeutic index in medicine. Thus, effective doses of a wide variety of anti-cancer agents are restricted by their non-selective, highly toxic effect on normal tissues. We proposed in DAMD17-98-1-8581 to design short peptides composed of two functional domains, one a tumor blood vessel 'homing' motif and the other a programmed cell death-inducing sequence, and synthesized them by simple peptide chemistry. The 'homing' domain was designed to guide the peptide to targeted cells and allow its internalization. The pro-apoptotic domain was designed to be non-toxic outside cells, but toxic when internalized into targeted cells by the disruption of mitochondrial membranes. Thus our approach was to create a non-toxic anticancer peptide that would only destroy tumor blood vessels while leaving normal blood vessels unharmed. We called these peptides **Hunter-Killer Peptides (HKP)**. We discuss here in our final report, and in Ellerby *et al.*, 1999 that we succeeded in the development of HKPs, demonstrating that although our 2 prototypes contained only 21 and 26 amino acid residues, they were selectively toxic to angiogenic endothelial cells and had strong anti-cancer activity in mice. Furthermore, we report here and in Arap *et al.*, 2002, that HKPs delayed the development of the cancers in prostate cancer-prone transgenic mice (TRAMP mice). Finally, as described in DAMA17-98-1-8581, we demonstrated the feasibility of using membrane-disrupting/pore-forming peptides as anti-neoplastic agents by evaluating the efficacy of a 69 amino acid peptide, **Small Globular Protein (SGP)**. As shown in our original preliminary results the anti-neoplastic effects of SGP on human breast carcinoma xenografts in nude mice were very dramatic. We now report here, and in a manuscript in preparation, that this peptide can reduce tumor volume (eliminating some tumors), and increase survival, in a xenograft model of human prostate carcinoma.

Body

Our work to date in designing, synthesizing, and testing Hunter-Killer Peptides (HKPs), and HKP-related peptides, is described in the accompanying reprints by Ellerby *et al.*, 1999, and Arap *et al.*, 2002, in the appendix. In the following, we re-state the approved **Statement of Work**, and then summarize how we have met our goals.

Statement of Work

Task 1. Design and synthesis of Hunter-Killer peptides (HKPs).

- We will use CNGRC peptide of Arap *et al.*, 1998 for the hunter domain for our initial studies.
- We will use 4-6 of the peptide designs of Javadpour *et al.*, 1996 for the killer domain to begin our studies.
- Both the all D- and all L-amino acid form (for the killer domain) of the HKPs will be made.
- The resulting HKPs will be synthesized and cyclized to HPLC purity by Anaspec (San Jose, CA).
- Depending upon the results of Task 2 and 3, we will proceed to optimize successful candidates, or search for new candidates through the use of function-based peptide design principles.

Goal- The overall goal of this task is to acquire HKPs for use in Task 2 and 3.

Task 2. Evaluate the efficacy and toxicity of the HKPs in vitro.

- We will test the HKPs toxicity to several mammalian cell lines, including 3T3s, through cell death studies in tissue culture.
- We will test the HKPs ability to disrupt mitochondrial membranes through swelling assays.
- The maximum concentration tolerated before any signs of mammalian cell death will be used with the minimum concentration required to swell mitochondria to compute a therapeutic index.

Goal- The overall goal of this task is to characterize the efficacy and toxicity of the HKPs and arrive at a therapeutic index, which then will act as a guide as to the concentrations of the HKPs to use in vivo.

Task 3. Evaluate the efficacy and toxicity of the HKPs in vivo.

- We will prepare tumors using human prostate cell lines.
- We will initiate tumor treatment studies with HKPs in nude mice and C57BL/6 mice. Treatments will be given locally, intraperitoneally or systemically. We will perform tumor volume, survival, and histopathological studies.
- Then we will test HKPs against primary and metastatic prostate cancer in the TRAMP transgenic mouse model of prostate cancer.
- We will perform tumor volume and histopathological studies to quantify the effect of HKPs on tumors and metastatic lesions. In addition, survival studies will be performed.

Final Summary of Results/Data:

We completed all our Tasks outlined in the Statement of Work, summarized here, and discussed further in the Key Accomplishments section.

Task 1

We completed Task 1, having developed 3 HKPs. The first two HKPs were described in Ellerby *et al.*, 1999, and the third in Arap *et al.*, 2002.

Task 2

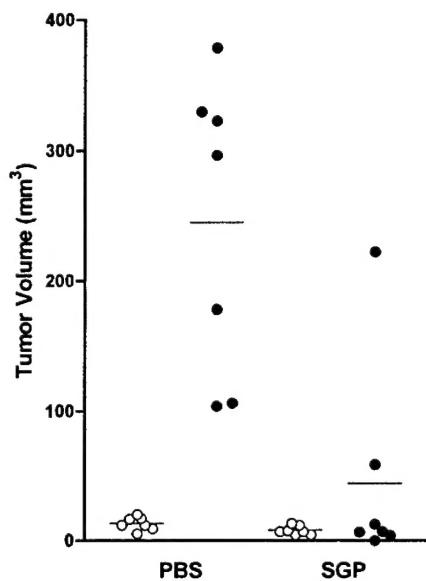
We completed Task 2, in Ellerby *et al.*, 1999, testing HKPs for efficacy and toxicity in tissue culture models of angiogenesis, mitochondria swelling studies, and in cell-free systems.

Task 3

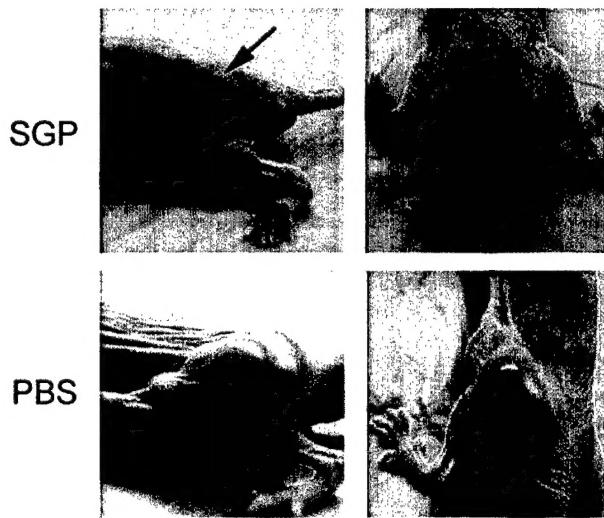
We completed Task 3 for xenografts of human breast carcinomas in Ellerby *et al.*, 1999. We also completed a study of HKP treatment of TRAMP mice, described in Arap *et al.*, 2002.

In addition to this published work we have evaluated the efficacy of an HKP related peptide SGP in xenografts of human prostate carcinomas. This manuscript is in preparation, and we summarize the data here. Human prostate carcinoma xenografts were established in nude mice

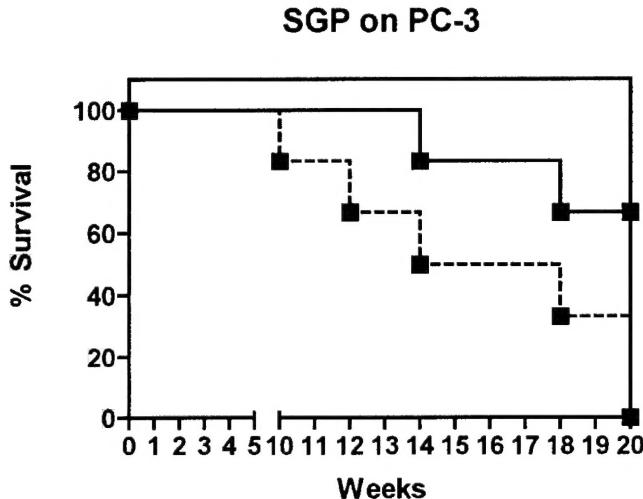
using the PC-3 cell line. After 4 weeks of growth, tumors were size matched and the mice were then given 1 weekly intratumoral injection of 100mM SGP for 14 weeks. Tumor volumes were measured at 0 and 10 weeks, before the control (PBS) animals began to die ($N = 7$). Individual tumor volumes are plotted below, with unfilled circles at $t = 0$ weeks, and filled circles at $t = 10$ weeks.



The tumor volumes for the SGP group were on average 6.25 times smaller than the control group (see below).



The SGP group also survived longer than the control group (see below).



We now plan to use SGP in combination with HKPs targeted to prostate tumor vasculature, and to normal prostate vasculature in combination therapies, and to continue to develop even less toxic HKPs. In this regard, in a pilot study, we have discovered that IP injections of HKPs are efficacious, and we are in the process of evaluating the IP injection route, both to calculate a new LD₅₀, and in the treatment of nude mice bearing human prostate carcinoma xenografts. In addition we are using the computer to design less toxic peptides, now accounting for the antibacterial effect of the whole peptide (including the targeting moiety).

Key Research Accomplishments

- (1) **We successfully designed and tested a Hunter-Killer Peptide, HKP-1, CNGRC-GG-D(KLAKLAK)₂.** We evaluated HKP-1 for efficacy and toxicity in a tissue culture model of angiogenesis developed by our laboratory. The *in vitro* therapeutic index, the ratio of [control peptide (50:50 mixture of uncoupled hunter and killer)]/[HKP] required to kill angiogenic endothelial cells, for HKP-1 was found to be 100. We also evaluated HKP-1 in a nude mouse model of human breast carcinoma. Tumor volumes were reduced and survival was extended. See accompanying reprint (Ellerby *et al.*, 1999, in the appendix).
- (2) **We successfully designed and tested a second more effective/less toxic Hunter-Killer Peptide, HKP-2, ACDCRGDCFC-GG-D(KLAKLAK)₂.** We substituted the targeting domain ACDCRGDCFC (Hunter) for the targeting domain CNGRC used in our prototype HKP-1. We evaluated HKP-2 for efficacy and toxicity in a tissue culture model of angiogenesis developed by our laboratory. The *in vitro* therapeutic index, the ratio of [control peptide (50:50 mixture of uncoupled hunter and killer)]/[HKP] required to kill angiogenic endothelial cells, for HKP-2 was found to be 4-5 times greater than that for HKP-1. We also evaluated HKP-2 in a nude mouse model of human breast carcinoma. Tumor volumes were reduced and metastasis was inhibited. See accompanying reprint (Ellerby *et al.*, 1999, in the appendix).
- (3) **We obtained new evidence in support of our hypothesized mechanism of HKP activity.** We demonstrated in our *in vitro* cell culture model of angiogenesis that HKPs are internalized into targeted endothelial cells, using 2 complementary experimental approaches (Ellerby

et al., 1999). First, we detected biotinylated homing peptides within targeted cells. Second, through the use of cell fractionation and mass spectrometry, we showed that HKPs are internalized, and concentrate in mitochondria (rather than say the nucleus) (Ellerby *et al.*, 1999). We also demonstrated *in vitro* that HKPs *disrupt the negatively charged membranes of mitochondria*, early in the apoptosis induction process, using 3 complementary experimental approaches. First, using mitochondria-specific fluorescent dye, we showed that mitochondria adopt an abnormal morphology (osmotic swelling) early in the cell death process, before any other obvious morphological change occurs that would be indicative of early apoptosis, such as “rounding-up.” Second, using electron microscopy, we confirmed that early on in the apoptosis of the targeted endothelial cells, mitochondria appear to be the only adversely affected membranous/organelar structure. Third, through the use of cell fractionation and mass spectrometry, we further confirmed that mitochondria are the target of HKPs.

(4) **We successfully tested a computer program designed to select optimum anti-mitochondrial peptides.** As proof of principle that anti-bacterial/mitochondrial peptides (killer domains) could be designed/chosen *in silico*, we created a computer program designed to select optimum mitochondrial membrane disrupting peptides. We have previously described a novel cancer chemotherapeutic approach based on the induction of apoptosis in targeted cells by homing pro-apoptotic peptides. In order to improve this approach we developed a computational method (approach for detecting potential apoptotic peptides, APAP) to detect short PAPs, based on the prediction of the helical content of peptides, the hydrophobic moment, and the isoelectric point. PAPs are toxic against bacteria and mitochondria, but not against mammalian cells when applied extracellularly. Among other peptides, substance P was identified as a PAP and subsequently demonstrated to be a pro-apoptotic peptide experimentally, and a disruptor of mitochondrial membranes. APAP thus provides a method to detect and ultimately improve pro-apoptotic peptides for chemotherapy. This work is described in del Rio *et al.*, 2001, in the appendix.

(5) **We successfully designed and tested a Hunter-Killer Peptide, SMSIARL-GG-_n(KLAKLAK)₂, in the TRAMP Mouse Model of Prostate Cancer.** This work was described in Arap *et al.*, 2002 [Arap, 2002 #2419].

(6) **We successfully designed and tested an HKP related peptide, SGP.** As described above, we successfully tested the anti-tumor effects of SGP in human prostate carcinoma xenografts in nude mice. The data show that SGP can reduce tumor volume and extend survival.

Reportable Outcomes

Papers, manuscripts, abstracts, presentations

(1) Ellerby HM, Arap W, Ellerby LM, Kain R, Andrusiak R, Del Rio G, Krajewski S, Lombardo CR, Rao R, Ruoslahti E, Bredesen DE, Pasqualini R (1999). “Anti-cancer activity of targeted pro-apoptotic peptides.” *Nature Medicine* 5(9):1032-1038.

(2) del Rio G, Castro-Obregon S, Rao R, Ellerby HM, Bredesen DE (2001). "APAP, a sequence-pattern recognition approach identifies substance P as a potential apoptotic peptide." *FEBS Lett.* 494(3):213-9.

(3) Arap W, Haedicke W, Bernasconi M, Kain R, Rajotte D, Krajewski S, Ellerby HM, Bredesen DE, Pasqualini R, Ruoslahti E (2002). "Targeting the prostate for destruction through a vascular address." *PNAS* 99(3):1527-31.

(4) Ellerby HM, Lee S, Andrusiak R, Ellerby LM, Chen S, Kiyota T, del Rio G, Sugihara G, Arap W, Bredesen DE & Pasqualini R (2002). "An artificially designed pore-forming protein with anti-tumor effects." In review *Nature Medicine*.

Patents and licenses applied for and/or issued

H. Michael Ellerby, Dale E. Bredesen, Renata Pasqualini, and Erkki Ruoslahti (1999). "Targeted Pro-Apoptotic Peptides for Cancer Therapy."

H. Michael Ellerby, Dale E. Bredesen, Renata Pasqualini, and Erkki Ruoslahti (1999). "Targeting the prostate for destruction through a vascular address."

Degrees obtained that are supported by this award

None.

Development of cell lines, tissue or serum repositories

None.

Informatics such as databases and animal models, etc

See ref. (2) above by del Rio et al.

Funding applied for based on work supported by this award

None at this time.

Employment or research opportunities applied for and/or received on experiences/training supported by this award

None at this time.

Conclusions

Importance and/or Implications

We have designed short peptides, Hunter Killer Peptides (HKP), composed of two functional domains, one a tumor blood vessel 'homing' motif and the other a programmed cell death-inducing sequence, and synthesized them by simple peptide chemistry. The 'homing' domain was designed to guide the peptide to targeted cells and allow its internalization. The pro-apoptotic domain was designed to be non-toxic outside cells, but toxic when internalized into targeted cells by the disruption of mitochondrial membranes. We demonstrated in Ellerby *et al.* 1999, that HKPs show strong anti-cancer activity in mice (xenografts of human breast carcinomas and melanomas). We then demonstrated in Arap *et al.*, 2002, that HKPs delayed the devel-

opment of the cancers in prostate cancer-prone transgenic mice (TRAMP mice). Finally, we demonstrated the feasibility of using membrane-disrupting/pore-forming peptides as anti-neoplastic agents by evaluating the efficacy of a 69 amino acid peptide, Small Globular Protein (SGP). The implications of this work are that man can engineer targeted and untargeted artificial peptides to be used systemically and locally as effective anti-cancer agents.

Knowledge as a scientific or medical product

The knowledge gained from these studies supported by the Department of Defense represents an important scientific and medical product in that HKPs are a new class of chemotherapeutic agents that can be modulated to obtain maximum therapeutic effect in the treatment of prostate cancer.

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Appendices

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ARTICLES

Anti-cancer activity of targeted pro-apoptotic peptides

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We have designed short peptides composed of two functional domains, one a tumor blood vessel 'homing' motif and the other a programmed cell death-inducing sequence, and synthesized them by simple peptide chemistry. The 'homing' domain was designed to guide the peptide to targeted cells and allow its internalization. The pro-apoptotic domain was designed to be non-toxic outside cells, but toxic when internalized into targeted cells by the disruption of mitochondrial membranes. Although our prototypes contain only 21 and 26 residues, they were selectively toxic to angiogenic endothelial cells and showed anti-cancer activity in mice. This approach may yield new therapeutic agents.

Tumor cell survival, growth and metastasis require persistent new blood vessel growth¹⁻³ (angiogenesis). Consequently, a strategy has emerged to treat cancer by inhibiting angiogenesis⁴. Peptides have been described that selectively target angiogenic endothelial cells⁵⁻⁸. Conjugates made from these peptides and the anti-cancer drug doxorubicin induce tumor regression in mice with a better efficacy and a lower toxicity than doxorubicin alone⁸. There is also a functional class of cell death-inducing receptors, or 'dependence receptors', which have embedded pro-apoptotic amino-acid sequences^{9,10}. These peptide domains are required for apoptosis induction by these receptors. The peptide fragments are thought to be released into the cytosol as cleavage products of caspase proteolysis, where they induce or potentiate apoptosis through unknown mechanisms^{9,10}. However, such peptides, and structurally similar pro-apoptotic antibiotic peptides, although they remain relatively non-toxic outside of eukaryotic cells, induce mitochondrial swelling and mitochondria dependent cell-free apoptosis^{10,11}.

There are more than 100 naturally occurring antibiotic peptides, and their *de novo* design has received much attention¹²⁻¹⁴. Many of these peptides are linear, cationic and α -helix-forming. Some are also amphipathic, with hydrophobic residues distributed on one side of the helical axis and cationic residues on the other¹⁵. Because their cationic amino acids are attracted to the head groups of anionic phospholipids, these peptides preferentially disrupt negatively charged membranes. Once electrostatically bound, their amphipathic helices distort the lipid matrix (with or without pore formation), resulting in the loss of membrane barrier function^{15,16}. Both prokaryotic cytoplasmic membranes and eukaryotic mitochondrial membranes (both the inner and the outer) maintain large transmembrane potentials, and have a high content of anionic phospholipids, reflecting

the common ancestry of bacteria and mitochondria¹⁵⁻¹⁹. In contrast, eukaryotic plasma membranes (outer leaflet) generally have low membrane potentials, and are almost exclusively composed of zwitterionic phospholipids^{16,18,20}. Many antibacterial peptides, therefore, preferentially disrupt prokaryotic membranes and eukaryotic mitochondrial membranes rather than eukaryotic plasma membranes.

If such nontoxic peptides were coupled to tumor targeting peptides that allow receptor-mediated internalization, the chimeric peptide would have the means to enter the cytosol of targeted cells, where it would be toxic by inducing mitochondrial-dependent apoptosis^{10,11}. Thus, we designed targeted pro-apoptotic peptides composed of two functional domains. The targeting domain was designed to guide the 'homing' pro-apoptotic peptides to targeted cells and allow their internalization^{8,21,22}. The pro-apoptotic domain was designed to be non-toxic outside of cells, but toxic when internalized into targeted cells by the disruption of mitochondrial membranes.

Design of the pro-apoptotic peptide

A computer-generated model and the sequence of one of our prototypes are shown in Fig. 1. For the targeting domain, we used either the cyclic (disulfide bond between cysteines) CNGRC peptide (Fig. 1) or the double-cyclic ACDCRGDCFC peptide (called RGD-4C), both of which have 'tumor-homing' properties^{5,8} and for which there is evidence of internalization^{8,21,22}. We synthesized this domain from all-L amino acids because of the presumed chiral nature of the receptor interaction. For the pro-apoptotic domain, we selected the synthetic 14-amino-acid peptide KLAKLAKKLAKLAK (Fig. 1), called (KLAKLAK)₂, because it killed bacteria at concentrations 1% of those required to kill eukaryotic cells¹³. We used the all-D enan-

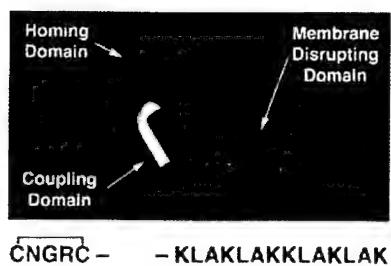


Fig. 1 Computer-generated model and amino-acid sequence of CNGRC-GG_n(KLAKLAK)₂. This peptide is composed of a 'homing' domain (blue) and a membrane-disrupting (pro-apoptotic) domain (red hydrophilic and green hydrophobic residues), joined by a coupling domain (yellow).

tiomer $\text{D}_n(\text{KLAKLAK})_2$ to avoid degradation by proteases^{12,23}. This strategy was possible because such peptides disrupt membranes by chiral-independent mechanisms^{23,24}. We coupled the targeting (CNGRC or RGD-4C) and pro-apoptotic $\text{D}_n(\text{KLAKLAK})_2$ domains with a glycylglycine bridge (Fig. 1) to impart peptide flexibility and minimize potential steric interactions that would prevent binding and/or membrane disruption.

D(KLAKLAK)2 disrupts mitochondrial membranes

We evaluated the ability of $\text{D}_n(\text{KLAKLAK})_2$ to disrupt mitochondrial membranes preferentially rather than eukaryotic plasma membranes by mitochondrial swelling assays, in a mitochondria-dependent cell-free system of apoptosis, and by cytotoxicity assays¹⁰. There was morphological evidence of damage to mitochondrial membranes by electron microscopy. The peptide $\text{D}_n(\text{KLAKLAK})_2$ induced considerable mitochondrial swelling at a concentration of 10 μM (Fig. 2a). Mild swelling was evident even at 3 μM (data not shown), 1% the concentration required to kill eukaryotic cells (approximately 300 μM), as determined by the lethal concentration required to kill 50% of a cell monolayer (LC_{50} ; Table 1). These results demonstrate that $\text{D}_n(\text{KLAKLAK})_2$ preferentially disrupts mitochondrial membranes rather than eukaryotic plasma membranes. Moreover, the peptide activated mitochondria-dependent cell-free apoptosis in a system composed of mitochondria suspended in cytosolic extract¹⁰, as measured by characteristic caspase-3-processing from an inactive zymogen to active protease²⁵ (Fig. 2b). A non- α -helix-forming peptide, DLSLARLATARLAI (negative control), did not induce mitochondrial swelling (Fig. 2a), was inactive in the cell-free system (Fig. 2b) and was not lethal to eukaryotic cells¹⁰. We also analyzed morphologic alterations in isolated mitochondria

by electron microscopy. The peptide $\text{D}_n(\text{KLAKLAK})_2$ induced abnormal mitochondrial morphology, whereas the control peptide DLSLARLATARLAI did not (Fig. 2c).

Targeted pro-apoptotic peptides induce apoptosis

We evaluated the efficacy and specificity of CNGRC-GG_n(KLAKLAK)₂ in KS1767 cells, derived from Kaposi sarcoma^{26,27} (Fig. 3a-d), and MDA-MB-435 human breast carcinoma cells^{5,8} (Table 1). We used KS1767 cells because they bind the CNGRC targeting peptide just as endothelial cells do. This may relate to the endothelial origin of the KS1767 cells²⁷. We used MDA-MB-435 cells as negative control cells because they do not bind the CNGRC targeting peptide⁸. Although CNGRC-GG_n(KLAKLAK)₂ was considerably toxic to KS1617 cells, an equimolar mixture of uncoupled CNGRC and $\text{D}_n(\text{KLAKLAK})_2$ (negative control), or $\text{D}_n(\text{KLAKLAK})_2$ alone, was much less toxic, indicative of a targeting effect (Table 1). In contrast, CNGRC-GG_n(KLAKLAK)₂ was not very toxic to MDA-MB-435 cells, which do not bind the CNGRC peptide (Table 1). The other targeted peptide (RGD-4C)-GG_n(KLAKLAK)₂, showed toxic effects similar to those of CNGRC-GG_n(KLAKLAK)₂ on KS1617 cells, whereas an equimolar mixture of uncoupled RGD-4C and $\text{D}_n(\text{KLAKLAK})_2$, used as a negative control, was not very toxic (Table 1; Fig. 3c-d).

Although evidence for internalization of CNGRC and RGD-4C into the cytosol of cells has been published^{5,8,21,22}, we directly demonstrated internalization using biotin-labeled peptides. CNGRC-biotin, but not untargeted CARAC-biotin, was internalized into the cytosol of cells (Fig. 3e-f). We also obtained direct evidence for internalization from experiments based on cell fractionation and mass spectrometry. CNGRC-GG_n(KLAKLAK)₂, but not CARAC-GG_n(KLAKLAK)₂, was indeed internalized and could be detected in mitochondrial as well as cytosolic fractions (data not shown).

Next, we evaluated the efficacy and specificity of CNGRC-GG_n(KLAKLAK)₂ in a tissue culture model of angiogenesis²⁸. During angiogenesis, capillary endothelial cells proliferate and migrate^{1,2}. Cord formation is a type of migration that can be studied *in vitro* by a change in endothelial cell morphology from the usual 'cobblestones' to chains or cords of cells²⁷. We tested the effect of CNGRC-GG_n(KLAKLAK)₂ on normal human dermal microvessel endothelial cells (DMECs) in the angiogenic conditions of proliferation and cord formation and in the angiostatic condition of a monolayer maintained at 100% confluence.

The treatment of DMECs with 60 μM CNGRC-GG_n(KLAKLAK)₂ led to a decrease in the percent viability over time compared with that of untreated controls, in the conditions of proliferation (Fig. 4a) or cord formation (Fig. 4b). In contrast, treatment with the untargeted peptide $\text{D}_n(\text{KLAKLAK})_2$ as a negative control led to a negligible loss in viability. Furthermore,

the LC_{50} for proliferating or migrating DMECs treated with CNGRC-GG_n(KLAKLAK)₂ was 10% of the LC_{50} for angiostatic DMECs maintained in a monolayer at 100% confluence (Table 1). This result indicates that CNGRC-GG_n(KLAKLAK)₂ kills cells in angiogenic but not angiostatic conditions. The LC_{50} for the untargeted control $\text{D}_n(\text{KLAKLAK})_2$ in angiogenic con-

Table 1 LC_{50} (μM) for eukaryotic cells treated with targeted pro-apoptotic peptides

	Angiostatic	Angiogenic		KS1767	MDA-MB-435
		Proliferation	Cord Form	Proliferation	Proliferation
$\text{D}_n(\text{KLAKLAK})_2$	492	346	368	387	333
CNGRC-GG _n (KLAKLAK) ₂	481	51*	34*	42*	415
(RGD-4C)-GG _n (KLAKLAK) ₂	-	-	-	10*	-

Results are means of three independent experiments. * $P<0.03$, t-test.

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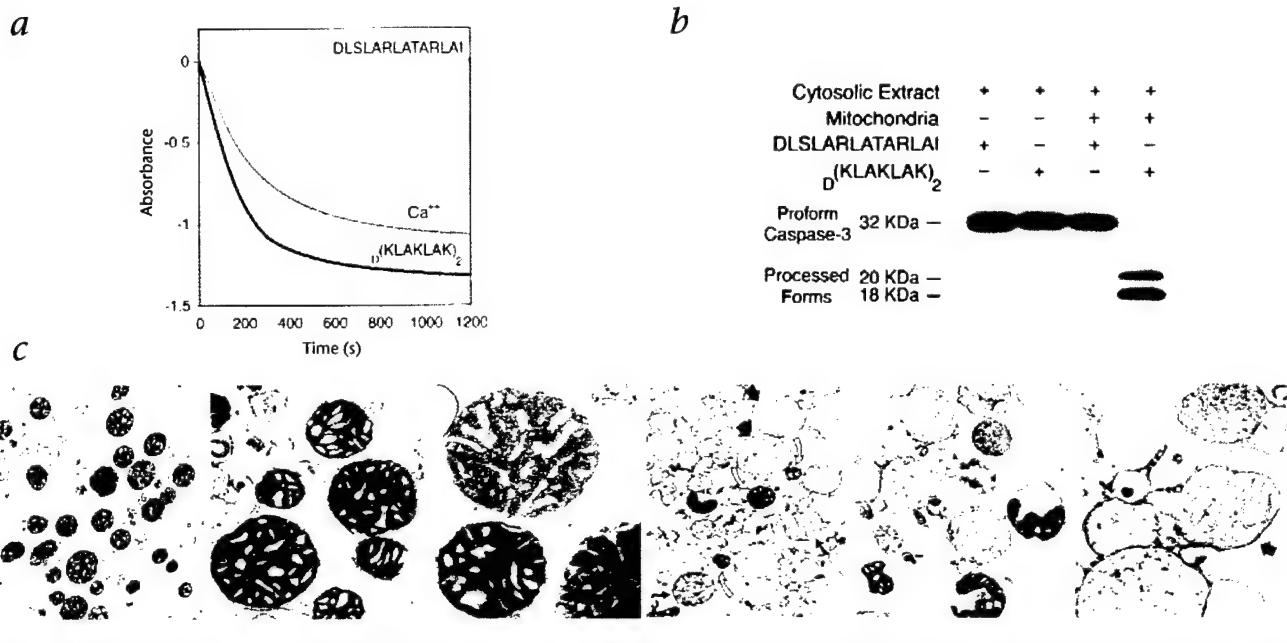


Fig. 2 $d(KLAKLAK)_2$ disrupts mitochondrial membranes. **a**, $d(KLAKLAK)_2$ or Ca²⁺ (positive control) induced mitochondrial swelling, whereas the non- α -helix-former DLSLARLATLAI (negative control) did not, as shown by mitochondrial swelling curves (optical absorbance spectrum). **b**, $d(KLAKLAK)_2$ activates cell-free apoptosis in a system composed of normal mitochondria and cytosolic extract, but DLSLARLATLAI does not. An immunoblot of caspase-3 cleavage from proform (32-kDa) to processed forms (18- and 20-kDa) demonstrates a mitochondria-dependent cell-free apoptosis (left margin, sizes). Results were reproduced in two independent experiments. **c**, Morphologic alterations in isolated mito-

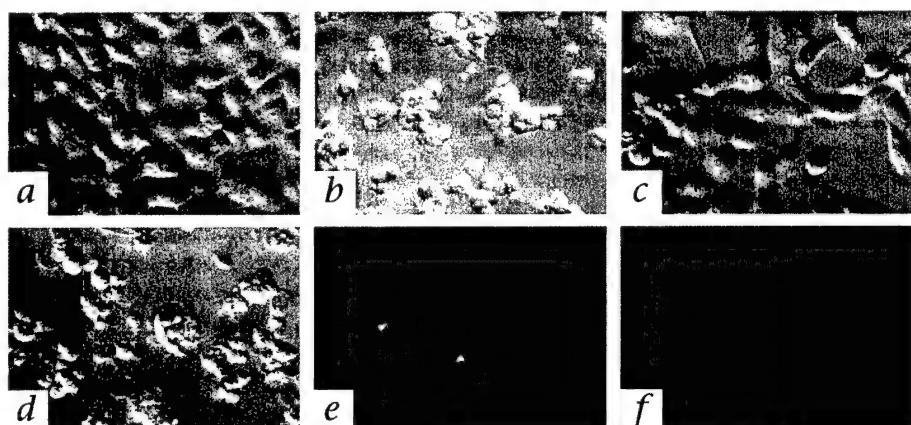
chondria analyzed by electron microscopy. Mitochondria incubated for 15 min with 3 μ M DLSLARLATLAI show normal morphology (left panels). In contrast, mitochondria incubated for 15 min with 3 μ M $d(KLAKLAK)_2$ show extensive morphological changes. The damage to mitochondria progressed from the stage of focal matrix resolution (short black arrow), through homogenization and dilution of condensed matrix content with sporadic remnants of cristae (long black arrows), to extremely swollen vesicle-like structures (thick black arrows; bottom right, higher magnification); few mitochondria had normal morphology (open arrows). Ultrathin sections are shown. Original magnification, $\times 4,000$ – $\times 40,000$.

ditions was similar to the LC₅₀ for CNGRC-GG- $d(KLAKLAK)_2$ under angiostatic conditions. An equimolar mixture of uncoupled $d(KLAKLAK)_2$ and CNGRC, a non-targeted form CARAC-GG- $d(KLAKLAK)_2$, and a 'scrambled' form, CGRNC-GG- $d(KLAKLAK)_2$, all gave results similar to those of $d(KLAKLAK)_2$.

We also studied the mitochondrial morphology of DMECs in the condition of proliferation, after treatment with 60 μ M CGRNC-GG- $d(KLAKLAK)_2$ or untargeted $d(KLAKLAK)_2$. The mitochondria in intact DMECs treated for 24 hours with the

equimolar mixture CNGRC and $d(KLAKLAK)_2$ remained morphologically normal (Fig. 4d), whereas those treated with CGRNC-GG- $d(KLAKLAK)_2$ showed altered mitochondrial morphology, evident in approximately 80% of cells (Fig. 4e), before the cells rounded-up. Ultimately, the DMECs treated with CNGRC-GG- $d(KLAKLAK)_2$ showed the classic morphological indicators of apoptosis, including nuclear condensation and fragmentation, as seen at 72 hours (Fig. 4f and g) (ref. 10). Apoptotic cell death (Fig. 4g) was confirmed with an assay for

Fig. 3 CNGRC-GG- $d(KLAKLAK)_2$ and (RGD-4C)-GG- $d(KLAKLAK)_2$ induce apoptosis. **a**, KS1767 cells treated with 100 μ M of non-targeted CARAC-GG- $d(KLAKLAK)_2$ (negative control) remain unaffected after 48 h. **b**, KS1767 cells treated with 100 μ M of CNGRC-GG- $d(KLAKLAK)_2$ undergo apoptosis, as shown at 48 h. Condensed nuclei and plasma membrane blebbing are evident. **c**, KS1767 cells treated with 10 μ M of an equimolar mixture of (RGD-4C) and $d(KLAKLAK)_2$ (negative control) remain unaffected after 48 h. **d**, KS1767 cells treated with 10 μ M of (RGD-4C)-GG- $d(KLAKLAK)_2$ undergo apoptosis, as shown at 48 h. Condensed nuclei and plasma membrane blebbing are evident. Scale bar represents 250 μ m. **e** and **f**, KS1767 cells treated with 100 μ M of CNGRC-biotin (**e**) or CARAC-biotin (**f**) for 24 h and subsequently



treated with streptavidin FITC demonstrate internalization of CNGRC-biotin, but not CARAC-biotin, into the cytosol.

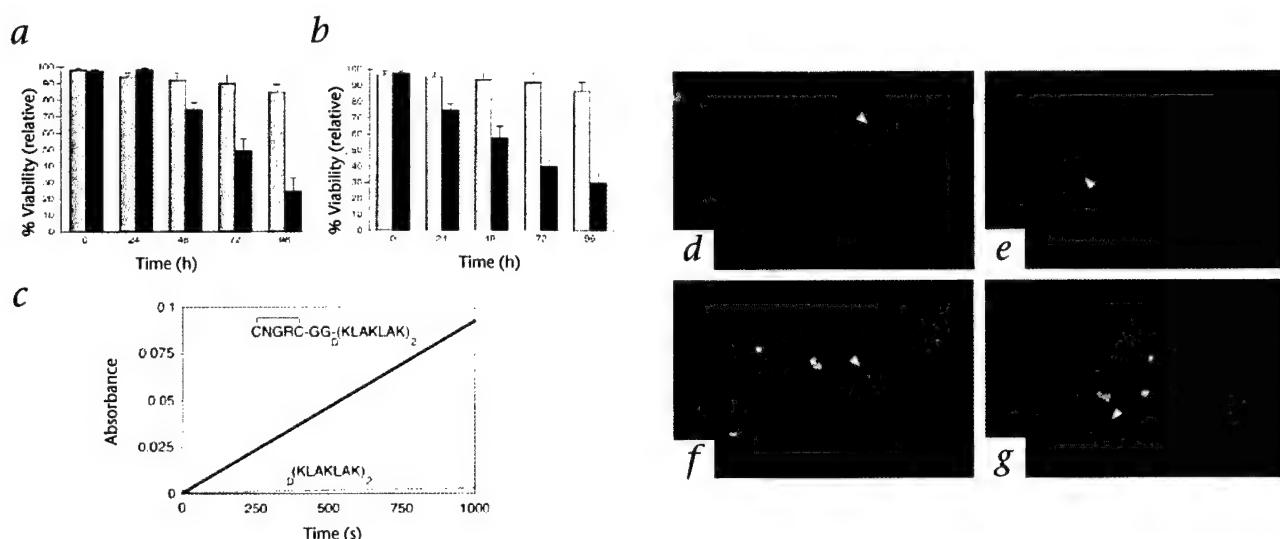


Fig. 4 CNGRC-GG- β (KLAKLAK) $_2$ induces apoptosis and mitochondrial swelling in DMECs. **a**, Proliferating DMECs treated with CNGRC-GG- β (KLAKLAK) $_2$ (filled bars) lose viability (apoptosis) over time ($P < 0.02$), but those treated with the control peptide β (KLAKLAK) $_2$ (gray bars) do not ($P < 0.05$). **b**, Cord-forming DMECs lose viability (apoptosis) over time (filled bars), but those treated with β (KLAKLAK) $_2$ (gray bars) do not ($P < 0.05$). **c**, Apoptotic cell death was confirmed with an assay for caspase 3 activity, as shown by the hydrolysis of DEVD-pNA with time. Results were reproduced in three independent experiments. **d**, Proliferating DMECs show normal nu-

clear (blue) and mitochondrial (red) morphology after 24 h of treatment with a mixture of 100 μ M β (KLAKLAK) $_2$ and CNGRC. **e–g**, Proliferating DMECs treated with 100 μ M CNGRC-GG- β (KLAKLAK) $_2$. After 24 h (**e**), cells show normal nuclear (blue) but abnormal mitochondrial (red) morphology. Mitochondrial swelling and dysfunction is shown by a decrease in fluorescence intensity and a change in morphology from an extended lace-like network to a condensed clumping of spherical structures. Classic morphological indicators of mid- to late apoptosis (for example, condensed and fragmented nuclei) are evident at 48 h (**f**) and 72 h (**g**) (arrow).

caspase 3 activity 10 . We also tested a caspase inhibitor for its effect on cell death induced by CNGRC-GG- β (KLAKLAK) $_2$. We used Kaposi sarcoma cells, as these cells bind CNGRC. The inhibitor zVAD.fmk, at a concentration (25 μ M) that inhibits caspases but not non-caspase proteases, inhibited the cell death induced by CNGRC-GG- β (KLAKLAK) $_2$ (data not shown). This result is compatible with the earlier demonstration that the

CNGRC-GG- β (KLAKLAK) $_2$ peptide is pro-apoptotic. Although the relatively early mitochondrial swelling is consistent with the putative mechanism of action, that is, a direct activation of the apoptotic machinery, we cannot rule out the possibility that the peptides actually kill by inducing some irreversible damage to cells which then activates the apoptotic program.

In addition to the fluorescence studies shown above, we studied cultured cells by electron microscopy to confirm that CNGRC-GG- β (KLAKLAK) $_2$ induces abnormal mitochondrial morphology in intact cells (Fig. 5). Kaposi sarcoma-derived KS1767 cells treated with the control peptide CARAC-GG- β (KLAKLAK) $_2$ for 72 hours showed no overall changes, with no or very minor changes in the mitochondria (Figs. 5a–c). In contrast, the mitochondria in KS1767 cells incubated for 12 hours

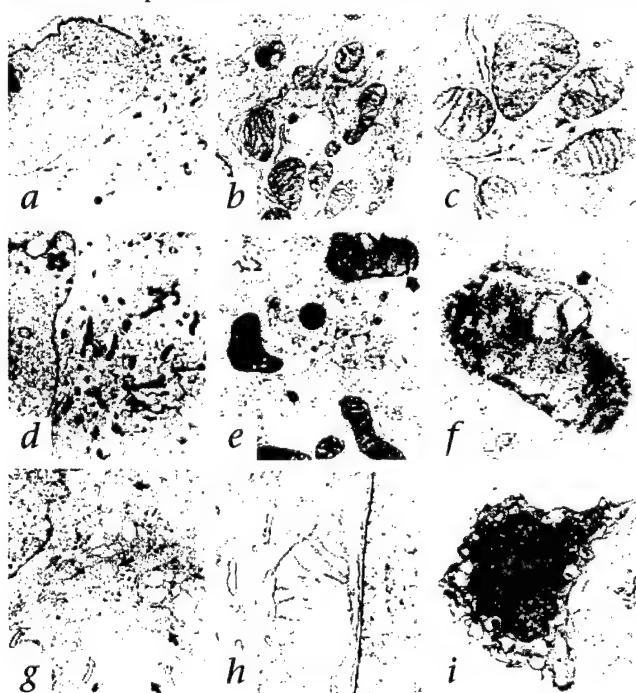
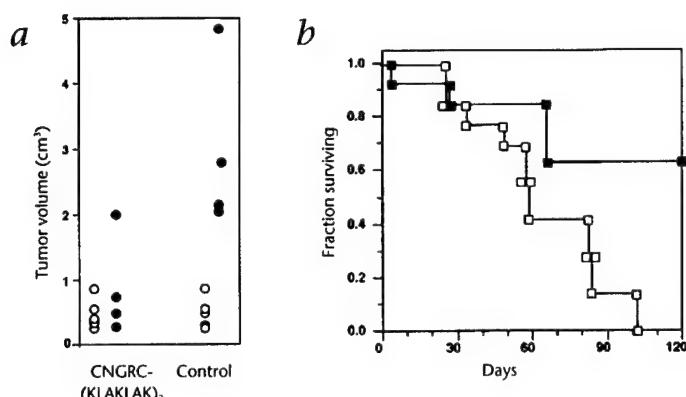


Fig. 5 Electron microscopic studies of cultured cells. **a–c**, KS1767 cells treated with 100 μ M CARAC-GG- β (KLAKLAK) $_2$ for 72 h show the representative ultrastructural details of normal cells, with no or negligible changes seen in the mitochondria. Original magnifications: **a**, $\times 4,000$; **b**, $\times 25,000$; **c**, $\times 45,000$. **d–f**, In contrast, the mitochondria in KS1767 cells incubated for 12 h with 100 μ M CNGRC-GG- β (KLAKLAK) $_2$ begin to show a condensed appearance and vacuolization despite a relatively normal cell morphology (black arrows). Original magnifications: **d**, $\times 12,000$; **e**, $\times 20,000$; **f**, $\times 45,000$. **g** and **h**, Progressive damage to KS1767 cells is evident after 24 h, when many mitochondria show typical large matrix compartments and prominent cristae, ultrastructural features of low level of oxidative phosphorylation. Original magnifications: **g**, $\times 12,000$; **h**, $\times 40,000$. Some of the swollen mitochondria (**g**, black arrows) are similar in appearance to those in isolated mitochondria treated with 100 μ M β (KLAKLAK) $_2$ (Fig. 2c, bottom right). **i**, In some cells, this process progressed to a final stage, with extensive vacuolization and the pyknotic, condensed nuclei typical of apoptosis. Original magnification, $\times 8,000$.

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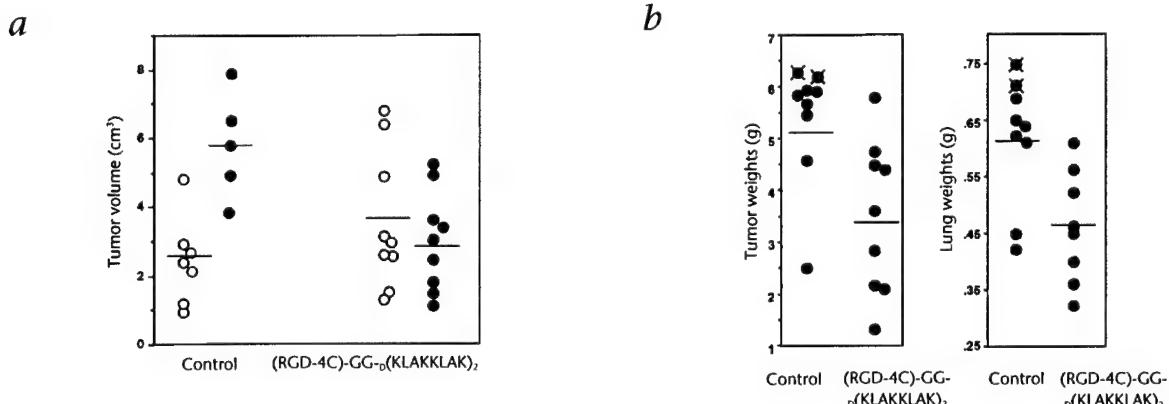
with CNGRC-GG-D(KLAKLAK)2 showed abnormal condensation and vacuolization despite a relatively preserved cell morphology (Fig. 5d-f, black arrows). Progressive cellular damage could be seen after 24 hours, when many mitochondria showed ultrastructural features of low-level oxidative phosphorylation (Fig. 5g and h); in later stages, some of the damaged mitochondria (Fig. 5g, black arrows) showed profound changes, as seen in the isolated mitochondria treated with D(KLAKLAK)2 (Fig. 2c, right lower panel). In some cells, this process progressed to a late apoptotic stage. Typical vacuolization and condensed nuclei became evident (Fig. 5i). These results show that the mitochondria underwent changes in morphology and function that were well-represented by a progression from a state of normal morphology and normal oxidative phosphorylation (Fig. 5a) to a state of condensed morphology and a high rate of oxidative phosphorylation (Fig. 5d) to a final edemic state (Fig. 5g) associated with a low energy level.

Treatment of nude mice bearing human tumor xenografts with CNGRC-GG-D(KLAKLAK)2 and (RGD-4C)-GG-D(KLAKLAK)2

Given our results in culture, we proceeded to test both targeted pro-apoptotic peptides *in vivo*, using nude mice with human MDA-MD-435 breast carcinoma xenografts. Tumor volume in the groups treated with CNGRC-GG-D(KLAKLAK)2 was on average 10% that of control groups (Fig. 6a); survival was also longer in these groups than in control groups (Fig. 6b). The control was a non-targeted 'mimic' CARAC-GG-D(KLAKLAK)2 peptide; the CARAC sequence has a charge, size and general structure similar to that of CNGRC. Some of the mice treated with CNGRC-GG-D(KLAKLAK)2 outlived control mice by several months, indicating that both primary tumor growth and metastasis were inhibited by CNGRC-GG-D(KLAKLAK)2. Treatment in nude mice bearing MDA-MD-435 breast carcinoma xenografts with (RGD-4C)-GG-D(KLAKLAK)2 also resulted in a significantly reduced tumor and metastatic burden (Fig. 7). Experimental parameters included tumor volumes before and after treatment (Fig. 7a), wet weights of the tumors (Fig. 7b, right) and weight of lung metastases (Fig. 7b, left). The control group was treated with an equimolar mixture of RGD-4C and D(KLAKLAK)2. Histopathological and TUNEL analysis showed cell death in the treated tumors and evidence of apoptosis and necrosis (data not shown).

To assess toxicity in mice without tumors, we have administered CNGRC-GG-D(KLAKLAK)2 or (RGD-4C)-GG-D(KLAKLAK)2 to both immunocompetent (balb/c) and to immunodeficient (balb/c nude) mice at a dose of 250 μ g/mouse per week for eight doses. No apparent toxicities have been found in 3 months. Moreover, in these conditions, the peptides are not immunogenic, as determined by ELISA of blood obtained from the immunocompetent mice (data not shown).

We have also evaluated the stability of the CNGRC-GG-D(KLAKLAK)2 and (RGD-4C)-GG-D(KLAKLAK)2 peptides *ex vivo* and in mice. We analyzed the two targeted peptides using mass spectrometry. In the first set of experiments, the targeted peptides were pre-mixed with whole blood and incubated at 37 °C.



assessed on day 1 (○) and day 90 (●). $P = 0.027$, *t*-test. **b**, Tumor weights (right) and lung metastatic burden (left) are also decreased in mice treated with (RGD-4C)-GG-D(KLAKLAK)2; these were measured when the experiment ended, on day 110 ($n = 9$ animals/group). $P < 0.05$, *t*-test.

The peptides were intact up to 1 hour in these conditions. In the second set of experiments, mice were injected intravenously with the two targeted peptides and blood samples were analyzed; the peptides were present at 10 minutes after administration (data not shown). We chose these short circulation times to coincide with the experimental conditions established for 'homing' of targeted peptides *in vivo*^{5,7,8}.

Targeted pro-apoptotic peptides represent a potential new class of anti-cancer agents; their activity may be optimized for maximum therapeutic effect by adjusting properties such as residue placement, domain length, peptide hydrophobicity and hydrophobic moment²⁹. Beyond this, future targeted pro-apoptotic peptides might be designed to disrupt membranes using a completely different type of pro-apoptotic domain such as β-strand/sheet-forming peptides³⁰. Our results provide a glimpse at a new cancer therapy combining two levels of specificity: 'homing' to targeted cells and selective apoptosis of such cells after entry.

Methods

Reagents. Human recombinant vascular endothelial growth factor (VEGF; PharMingen, San Diego, California), antibody against caspase-3 (Santa Cruz Biotechnology, Santa Cruz, California), streptavidin FITC (Sigma) and N-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA; BioMol, Plymouth Meeting, Pennsylvania) were obtained commercially. Peptides were synthesized to our specifications at greater than 90% purity by HPLC (DLSLARLATARLAI, Coast (San Diego, California); all other peptides, AnaSpec (San Jose, California).

The computer-generated model was made with Insight II (Molecular Simulations, San Diego, California) running on an O₂ work station (Silicon Graphics, Mountain View, California)

Cell culture. Dermal microvessel endothelial cells (DMECs) were grown in CADMEC Growth Media™ (media and cells from Cell Applications, San Diego, California). DMECs were then cultured in three experimental conditions: proliferation (30% confluence in a growth media supplemented with 500 ng/ml VEGF); no proliferation (100% confluence in media formulated to maintain a monolayer); and cord formation (60% confluence (required for induction) in media formulated to induce cord formation). KS1767 and MDA-MB-435 cells were cultured as described^{5,8,27,28}.

Internalization assay. KS1767 cells grown on coverslips were treated with 100 μM biotin-labeled CNGRC or biotin-labeled CARAC (negative control) for 24 h. Streptavidin FITC was added to the coverslips, and cells were then viewed on an inverted microscope (Nikon TE 300) using a FITC filter.

Mitochondrial swelling assays. Rat liver mitochondria were prepared as described¹⁰. The concentrations used were 10 μM $\text{o}(\text{KLAKLAK})_2$, 10 μM DL-SLARLATARLAI (negative control), or 200 μM Ca²⁺ (positive control). The peptides were added to mitochondria in a cuvette, and swelling was quantified by measuring the optical absorbance at 540 nm.

Cell-free apoptosis assays. Cell-free systems were reconstituted as described¹⁰. For the mitochondria-dependent reactions, rat liver mitochondria were suspended in normal (non-apoptotic) cytosolic extracts of DMECs. The peptides were added at a concentration of 100 μM. After incubation for 2 h at 30 °C or 37 °C, mitochondria were removed by centrifugation, and the supernatant was analyzed by SDS-PAGE and immunoblotting (12% gels, BioRad, Richmond, California). Proteins were transferred to PVDF membranes (BioRad, Richmond, California) and incubated with antibody against caspase-3, followed by ECL detection (Amersham).

Caspase activity of cell lysates. The caspase activity of DMEC lysates was measured as described¹⁰. Aliquots of cell lysates (1 μl lysate; 8–15 mg/ml) were added to 100 μM DEVD-pNA (100 μl; 100 mM HEPES, 10% sucrose, 0.1% CHAPS and 1 mM DTT, pH 7.0). Hydrolysis of DEVD-pNA was monitored by spectrophotometry (400 nm) at 25 °C.

Morphological quantification of cellular apoptosis. Percent viability and LC₅₀ (Table 1) were determined by apoptotic morphology¹⁰. For the percent viability assay, DMECs were incubated with 60 μM active peptide or control peptide. Cell culture medium was aspirated at various times from adherent cells, and the cells were gently washed once with PBS at 37 °C. Then, a 20-fold dilution of the dye mixture (100 μg/ml acridine orange and 100 μg/ml ethidium bromide) in PBS was gently pipetted on the cells, which were viewed on an inverted microscope (Nikon TE 300). The cell death seen was apoptotic cell death and was confirmed by a caspase activation assay. Not all cells progressed through the stages of apoptosis at the same time. At the initial stages, a fraction of the cells were undergoing early apoptosis. At later stages, this initial fraction had progressed to late apoptosis and even to the necrotic-like stage associated with very late apoptosis (for example, loss of membrane integrity in apoptotic bodies). However, these cells were joined by a new fraction undergoing early apoptosis. Thus, cells with nuclei showing margination and condensation of the chromatin and/or nuclear fragmentation (early/mid-apoptosis; acridine orange-positive) or with compromised plasma membranes (late apoptosis; ethidium bromide-positive) were considered not viable. At least 500 cells per time point were assessed in each experiment. Percent viability was calculated relative to untreated controls. LC₅₀ for monolayer, proliferation (60% confluence), and cord formation were assessed at 72 h.

Mitochondrial morphology. DMECs after 24 and 72 h of treatment with peptide were incubated for 30 min at 37 °C with a mitochondrial stain (100 nM MitoTracker Red™ CM-H₂XROS; the nonfluorescent, reduced form of the compound) and a nuclear stain (500 nm DAPI; Molecular Probes, Eugene, Oregon). Mitochondria were then visualized under fluorescence microscopy (100x objective) under an inverted microscope using a triple wavelength filter set (Nikon).

Electron microscopy. Rat liver mitochondria were prepared as described¹⁰. The mitochondria were incubated either with a control peptide (DL-SLARLATARLAI) or with 3 μM $\text{o}(\text{KLAKLAK})_2$. The effects of the treatment were assessed at different times (Fig. 2c). Kaposi sarcoma cells were collected from 24-well Biocoat Cell culture inserts for electron microscopy (Becton Dickinson, Franklin Lakes, New Jersey). Cell monolayers at 80% confluence were exposed to either 100 μM CARAC-GG- $\text{o}(\text{KLAKLAK})_2$ (control) or CNGRC-GG- $\text{o}(\text{KLAKLAK})_2$ (targeted) (Fig. 5). All specimens were fixed with 3% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.4 for 30 to 45 min at the room temperature, followed by postfixation with aqueous 1% osmium tetroxide and 2% uranyl acetate. After dehydration using a graded series of ethanol rinses, tissues were embedded in resin. Ultrathin sections after additional counterstainings were viewed and photographed on an electron microscope (Hitachi H-600).

Human tumor xenografts. MDA-MB-435-derived tumor xenografts were established in female nude mice 2 months old (Jackson Labs, Bar Harbor, Maine) as described³¹. The mice were anesthetized with Avertin as described³¹. The peptides were administered at a dose of 250 μg/week per mouse, given slowly through the tail vein in a volume of 200 μl. Three-dimensional measurements of tumors were made by caliper on anesthetized mice, and were used to calculate tumor volume^{5,8}. Then, tumors and lungs were surgically removed and the wet weights recorded. Animal experimentation was reviewed and approved by the Institute's Animal Research Committee.

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Research article

Suppression of murine collagen-induced arthritis by targeted apoptosis of synovial neovasculature

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Abstract

Because angiogenesis plays a major role in the perpetuation of inflammatory arthritis, we explored a method for selectively targeting and destroying new synovial blood vessels. Mice with collagen-induced arthritis were injected intravenously with phage expressing an RGD motif. In addition, the RGD peptide (RGD-4C) was covalently linked to a proapoptotic heptapeptide dimer, D₁KLAKLAK₂, and was systemically administered to mice with collagen-induced arthritis. A phage displaying an RGD-containing cyclic peptide (RGD-4C) that binds selectively to the $\alpha\beta 3$ and $\alpha\beta 5$ integrins accumulated in inflamed synovium but not in normal synovium. Homing of RGD-4C phage to inflamed synovium was inhibited by co-administration of soluble RGD-4C. Intravenous injections of the RGD-4C-D₁KLAKLAK₂ chimeric peptide significantly decreased clinical arthritis and increased apoptosis of synovial blood vessels, whereas treatment with vehicle or uncoupled mixture of the RGD-4C and the untargeted proapoptotic peptide had no effect. Targeted apoptosis of synovial neovasculature can induce apoptosis and suppress clinical arthritis. This form of therapy has potential utility in the treatment of inflammatory arthritis.

Keywords: angiogenesis, apoptosis, collagen-induced arthritis, rheumatoid arthritis

Introduction

In rheumatoid arthritis (RA), the synovium is characterized by hyperplasia of the intimal lining and mononuclear infiltration of the sublining, leading to erosion of cartilage and subchondral bone by invasive pannus [1]. Angiogenesis plays a crucial role in the formation of pannus, and the extensive network of blood vessels facilitates recruit-

ment of mononuclear cells [2]. Proangiogenic mediators found in RA synovial tissue regulate migration and proliferation of endothelial cells. In addition, $\alpha\beta 3$ and $\alpha\beta 5$ integrins are important in angiogenesis [3–5]. $\alpha\beta 3$ is expressed on synovial blood vessels in rheumatoid arthritis [6,7], and $\alpha\beta$ antagonists injected directly into the joint suppress synovitis in rabbits [7]. Therefore, targeted

BSA = bovine serum albumin; CIA = collagen-induced arthritis; DMEM = Dulbecco's modified Eagle's medium; NMR = nuclear magnetic resonance; PBS = phosphate-buffered saline [solution]; RA = rheumatoid arthritis; RGD-4C = the peptide CDCRGDCFC; TU = transducing units; TUNEL = transferase-mediated UTP end labeling.

induction of apoptosis in the neovasculature is a potential therapy for RA [8].

We evaluated a novel method of targeted drug delivery to inflamed joints using peptides that selectively bind to neovasculature [9]. Our data show that a constrained cyclic RGD peptide that binds to $\alpha\beta 3$ and $\alpha\beta 5$ integrins [10] homes to blood vessels in inflamed synovium after systemic administration. This peptide was covalently linked to a 14-amino-acid proapoptotic peptide and successfully suppressed arthritis in collagen-induced arthritis (CIA).

Materials and methods

Phage and peptides

Insertless fd phage and phage presenting the peptide CDCRGDCFC (RGD-4C) was prepared as previously described elsewhere [10–12]. The chemical structure of RGD-4C was determined by NMR analysis and is described in detail elsewhere [13]. K91kan bacteria were a gift from G Smith. The peptides RGD-4C, CARAC, $D_{(KLAKLAKKLAKLAK)}$ [designated $D_{(KLAKLAK)}_2$], and CDCRGDCFC-GG- $D_{(KLAKLAKKALKLAK)}$ [designated RGD-4C- $D_{(KLAKLAK)}_2$] were synthesized by AnaSpec, Inc (San Jose, CA, USA).

Collagen-induced arthritis in mice

CIA was induced in 6- to 8-week-old male DBA/1J mice (Jackson Laboratory, Bar Harbor, ME, USA), as previously described [14,15].

Phage delivery and detection

To determine homing characteristics of the RGD-4C phage *in vivo*, mice with CIA on day 35 after immunization were anesthetized and injected with 200 μ l of medium containing 10^{10} transducing units (TU) of phage into the tail vein. After 5 min, the mice were perfused with 5 ml of medium given through the right atrium. The organs of interest and synovia were pooled, homogenized, incubated with K91kan bacteria for 30 min, and plated on tetracycline plates. Phage enrichment was calculated as the ratio of TU/g of synovial tissue divided by the TU/g of brain tissue. Because animals are perfused and sacrificed within minutes after administration, relative rates of penetration into the target tissue do not influence this assay system (i.e., the target is the intravascular surface of the blood vessels). For the competitive peptide inhibition studies, the phage was injected with either 1 mg of the RGD-4C peptide or with 1 mg of a control peptide, CARAC [16]. Unimmunized mice were used as normal controls.

Treatment protocol for CIA

In the clinical efficacy study, mice with established CIA were anesthetized and injected intravenously on days 35 and 41 with either 500 μ g of covalently linked RGD-4C- $D_{(KLAKLAK)}_2$ dissolved in 500 μ l DMEM ($N=14$), with a mixture of the equimolar amounts of uncoupled RGD-4C

and $D_{(KLAKLAK)}_2$ dissolved in 500 μ l DMEM ($N=20$), or with 500 μ l DMEM only ($N=20$). The clinical arthritis scores were assessed daily in a blinded manner using a semiquantitative scoring system from 0 to 4+ for each paw (maximum score) [14,15].

Immunohistochemical staining and antibodies

Mice were sacrificed on day 35 after immunization and their paws and internal organs were fixed in a 10% formalin solution for 24 hours, decalcified, and embedded in paraffin. The primary antibodies (horseradish peroxidase/anti-M13 monoclonal conjugate [Pharmacia Biotech, Piscataway, NJ, USA], rabbit anti- $\alpha\beta$ antibody [17]) and secondary antibody (swine anti-rabbit-AP; Dako, Glostrup, Denmark) were diluted in 2% BSA-PBS. Peroxidase activity was detected using 3,3'-diaminobenzidine, and alkaline phosphatase activity was detected using alkaline phosphatase kit III (Vector Laboratories Inc, Burlingame, CA, USA).

Terminal deoxynucleotidyl transferase-mediated UTP end labeling (TUNEL) assay

The paws of the animals treated with vehicle or 500 μ g of RGD-4C coupled to $D_{(KLAKLAK)}_2$ were fixed in a 10% formalin solution, decalcified for 14 days in a 15% EDTA-PBS solution at 4°C, and embedded in paraffin. Sections (5 μ m) were incubated with proteinase K (20 μ g/ml) for 20 minutes. The *In situ* Death Detection Kit from Boehringer Mannheim GmbH (Mannheim, Germany) was used in accordance with the manufacturer's instructions.

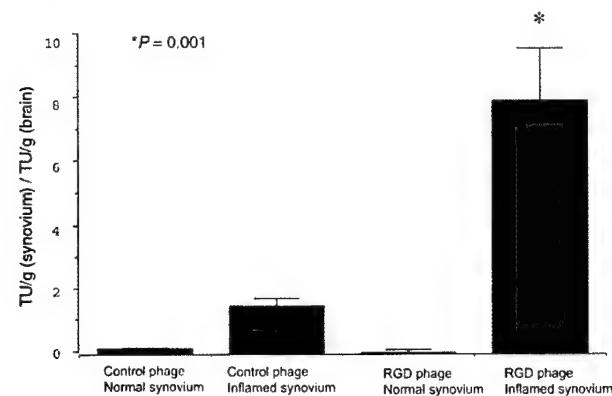
Results

Phage displaying RGD-4C accumulates in inflamed synovium

RGD-4C phage was injected intravenously into DBA/1J mice with active CIA on day 35 ($N=18$). Control mice with CIA received insertless fd phage ($N=14$). After circulation of the phage and perfusion with medium, the control organ (the brain) and synovium from arthritic joints were surgically dissected and phage enrichment was calculated as the ratio of TU/g of synovial tissue to TU/g of control tissue. Accumulation of the RGD-4C phage in inflamed synovium was 8.0 ± 1.7 (mean \pm standard deviation) fold that in brain ($P<0.01$). For the control phage, this value was 1.5 ± 0.3 (Fig. 1). Neither the RGD-4C phage nor the control phage accumulated in synovium of normal DBA/1J mice ($N=6$ and 5, respectively; $P<0.0001$ compared with inflamed synovium). Other organs (lung, liver, spleen, heart, kidney, pancreas, and gut) were also tested for enrichment of the RGD-4C and control phage. There was no selective accumulation of the RGD-4C phage in these organs (data not shown).

Inhibition of RGD-4C phage by a competing peptide

After showing that the RGD-4C phage targets inflamed synovium, we assessed the specificity of homing using the soluble RGD-4C peptide, a competitive inhibitor. The

Figure 1

Phage homing to inflamed synovium. DBA/1J mice with ('inflamed') and without ('normal') collagen-induced arthritis were injected intravenously on day 35 with 10^{10} transducing units (TU) of phage expressing RGD-4C on their surface and control phage. Phage enrichment was calculated as the ratio of TU/g in synovial tissue to TU/g in brain tissue. RGD-4C phage accumulate in inflamed synovium, but not in normal synovium ($P < 0.0001$).

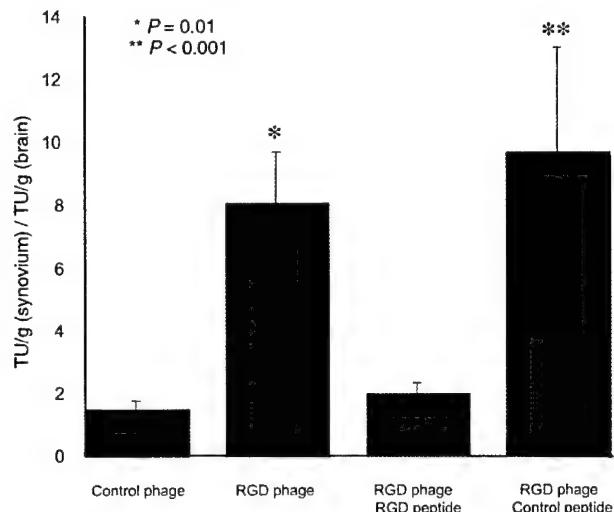
RGD-4C phage was co-injected with 1 mg of either the RGD-4C peptide ($N = 5$) or a control peptide (CARAC; $N = 5$). Figure 2 shows that soluble RGD-4C peptide, but not the control peptide, blocked accumulation of the RGD-4C phage in inflamed synovium.

RGD-4C-expressing phage and $\alpha\beta$ co-localize in synovial blood vessels

The localization of RGD-4C phage in synovium was examined in more detail by immunohistochemistry. Staining of paraffin-embedded sections revealed localization of RGD-4C-expressing phage to small vessels in the sublining of the inflamed synovium (Fig. 3a). Expression of the integrins containing $\alpha\beta$ was also observed in the small vessels in the inflamed synovial sublining (Fig. 3b). Immunohistochemical analysis did not reveal phage in the synovium of normal mice injected with the RGD-4C phage.

Suppression of arthritis by targeted apoptosis of synovial neovasculature

DBA/1J mice with CIA were administered a chimeric compound consisting of the peptide RGD-4C covalently linked to the proapoptotic peptide $\text{D}_5(\text{KLAKLAK})_2$ [16]. The chimeric compound was injected intravenously on days 35 and 41. Control mice with CIA were either injected with the vehicle or were co-injected with similar quantities of the uncoupled RGD-4C and $\text{D}_5(\text{KLAKLAK})_2$ peptides. The mean pretreatment score was 10.3 ± 0.5 . Neither the vehicle nor the uncoupled mixture of RGD-4C with $\text{D}_5(\text{KLAKLAK})_2$ had no effect on arthritis, whereas the targeted chimeric compound significantly decreased clinical arthritis on day 44 ($P < 0.001$; Fig. 4).

Figure 2

Inhibition of RGD-4C phage homing with the cognate soluble peptide. In competitive peptide inhibition studies in mice with collagen-induced arthritis, RGD-4C phage ('RGD phage') was co-injected with 1 mg of either RGD-4C peptide or control peptide (CARAC). Note accumulation of RGD-4C phage in inflamed synovium ($P < 0.01$).

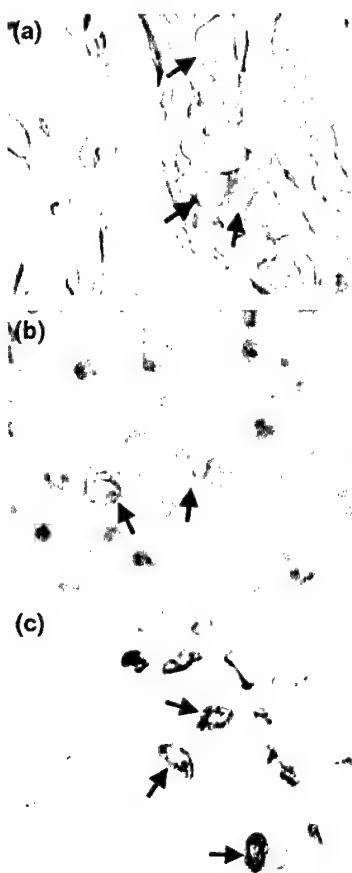
TUNEL studies were performed on joint specimens collected 72 hours after intravenous injection of the chimeric peptide. Abundant positive cells were detected in small vessels of the inflamed synovium (Fig. 3c) but not in synovium of arthritic mice injected with vehicle (not shown). Positive cells were not found in lung, liver, or spleen of chimera-treated arthritic mice, indicating that apoptosis was selective to inflamed synovium.

Discussion

Angiogenesis has been implicated in inflammatory diseases such as RA [2,8]. Synovial tissues from patients with RA express more of the angiogenesis marker, $\alpha\beta 3$ integrin, than control synovium [6,7,18]. An angiostatic compound suppressed arthritis in rat adjuvant arthritis and CIA [19,20], and intra-articular administration of an RGD peptide that binds to $\alpha\beta 3$ decreased synovial inflammation in a rabbit model of arthritis [7].

The homing properties of the cyclic RGD peptide allowed us to deliver a toxic agent – the proapoptotic peptide $\text{D}_5(\text{KLAKLAK})_2$ – to the synovial microvasculature through systemic administration. Homing minimized systemic toxicity in this model, as has been found in experimental tumor treatments [9,16]. Systemic treatment with RGD peptides that are selective for $\alpha\beta 3$ reduces angiogenesis, presumably by blocking the function of the integrin [21]. However, the systemic administration of free RGD-4C peptide had no effect on arthritis, indicating that the RGD-4C merely acts to target the proapoptotic peptide.

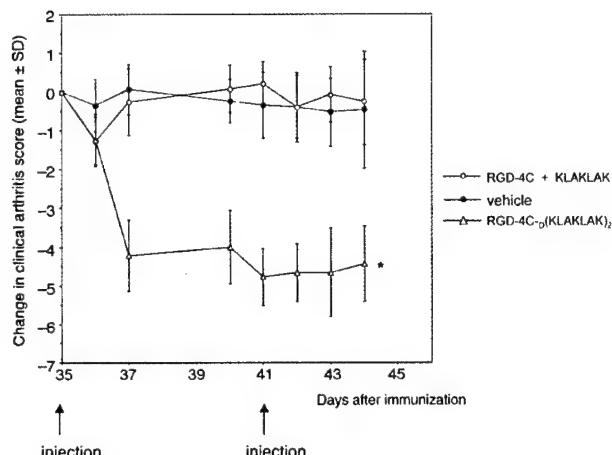
Figure 3



Detection of RGD-4C-phage, $\alpha\beta 3$, and TUNEL positive cells in synovial blood vessels. Mice with established collagen-induced arthritis were injected with RGD-4C and sections were stained for (a) phage (blue blood vessels, denoted here by arrows) or (b) $\alpha\beta 3$ (brown blood vessels, denoted here by arrows) by immunohistochemistry. In a second experiment, mice with collagen-induced arthritis were given RGD-4C- $\text{D}_0(\text{KLAKLAK})_2$ on day 35 and TUNEL assays were performed on day 38. Positive cells were found in sublining vessels of inflamed synovium in the animals treated with RGD-4C- $\text{D}_0(\text{KLAKLAK})_2$ (arrows) (c) but not in the control animals (not shown).

The RGD-4C peptide also enhances internalization of RGD-4C- $\text{D}_0(\text{KLAKLAK})_2$ [9]. When covalently linked to the homing peptide RGD-4C, the proapoptotic peptide enters cells that express $\alpha\beta 3$ or $\alpha\beta 5$ [22]. Once in the cytoplasm, it disrupts mitochondrial membranes and initiates apoptosis [15]. Distribution studies *in vivo* followed by cell fractionation indicate that RGD-4C- $\text{D}_0(\text{KLAKLAK})_2$ localizes to mitochondrial membranes of the target cells but not to endothelial cells in control tissues [15]. Cells that do not express these integrins are spared, because the proapoptotic peptide alone is not internalized. As with our studies, Pasqualini and colleagues showed that αv -directed RGD-

Figure 4



Changes in clinical arthritis scores in mice with collagen-induced arthritis after treatment with peptides or the vehicle. Mice were injected intravenously on days 35 and 41 with either the targeted proapoptotic chimeric peptide RGD-4C- $\text{D}_0(\text{KLAKLAK})_2$ or a mixture of the uncoupled peptides RGD-4C and $\text{D}_0(\text{KLAKLAK})_2$ or the vehicle. Clinical arthritis scores were evaluated using a scale of 0 to 4+ for each paw (maximum = 16). The mean pretreatment score was 10.3 ± 0.5 .

4C phage is not detected in normal tissues, but accumulates on the surface of tumor blood vessels [23].

Intravenous treatment of mice with established arthritis using RGD-4C- $\text{D}_0(\text{KLAKLAK})_2$ induced apoptosis of endothelial cells in the inflamed synovium. This was associated with decreased severity of arthritis compared with untreated mice or mice treated with a mixture of uncoupled RGD-4C and $\text{D}_0(\text{KLAKLAK})_2$ peptides. Since we did not observe systemic toxicity and did not find apoptotic cells in other tissues besides the inflamed synovium, RGD-4C- $\text{D}_0(\text{KLAKLAK})_2$ appears to have a favorable pharmacological profile.

These data support the feasibility of administering systemic agents that home to sites of inflammation as a means of delivering a therapeutic agent. This approach can treat multiple sites simultaneously while not accumulating in normal tissues. Our study is the first example of selective proapoptotic treatment of an inflammatory disease using homing peptides and could potentially be used to deliver therapeutic agents to inflamed joints.

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APAP, a sequence-pattern recognition approach identifies substance P as a potential apoptotic peptide

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Abstract We have previously described a novel cancer chemotherapeutic approach based on the induction of apoptosis in targeted cells by homing pro-apoptotic peptides. In order to improve this approach we developed a computational method (approach for detecting potential apoptotic peptides, APAP) to detect short PAPs, based on the prediction of the helical content of peptides, the hydrophobic moment, and the isoelectric point. PAPs are toxic against bacteria and mitochondria, but not against mammalian cells when applied extracellularly. Among other peptides, substance P was identified as a PAP and subsequently demonstrated to be a pro-apoptotic peptide experimentally. APAP thus provides a method to detect and ultimately improve pro-apoptotic peptides for chemotherapy. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Apoptosis; Antibacterial peptide; Bioinformatics

1. Introduction

We have previously described the finding that an antibacterial peptide, when targeted intracellularly to the angiogenic vasculature (i.e. to the endothelial cells) supplying tumors, can induce apoptosis by swelling their mitochondria [1], leading to the loss of tumor blood supply and consequent tumor regression. We named these chemotherapeutic peptides homing pro-apoptotic peptides. We designed the pro-apoptotic part of the peptides to induce endothelial cell apoptosis through mitochondrial swelling. The peptides are positively charged and the mitochondria, like bacteria, have negatively charged membranes, thus the peptides are attracted to and disrupt the mitochondrial membrane [2,3]. The initial results were obtained with a 21-residue peptide, of which the carboxy-terminal 14 amino acids represented the pro-apoptotic peptide, with the amino-terminal seven amino acids comprising the targeting peptide and a glycylglycine bridge. The therapeutic index (TI) of the initial pro-apoptotic peptides is approximately 10. In order to increase the TI and minimize

the length of these peptides, we designed a computational approach to detect short, linear and specific pro-apoptotic peptides.

Apoptosis in mammals and other eukaryotic organisms is a characteristic process of cell death, which can, among its other effects, limit the spread of viruses and other intracellular organisms [4]. For example, the difference in viral titer during baculoviral infection with and without apoptosis inhibition is 200–15 000-fold [4]. Thus apoptosis is a mechanism of defense against pathogenic infections.

Apoptosis proceeds by the activation of a group of cysteine proteases called caspases [5]. One of these, caspase-9, is activated when cytochrome *c* is released from mitochondria, which may occur with the disruption of the mitochondrial outer membrane [6]. This cytochrome *c* release in apoptotic cells may be induced by pro-apoptotic members of the Bcl-2 family, such as Bax and Bid, although the mechanism by which this is achieved is incompletely understood [7]. Nonetheless, the similarities between bacterial and mitochondrial membranes (and membrane potentials) suggested the possibility that there may be similarities between the effect of the antibacterial/pro-apoptotic peptides and pro-apoptotic Bcl-2 family members.

Antibacterial peptides in multicellular organisms are thought to serve as a defense against microbial pathogens. Originally found in invertebrates, antibacterial peptides have now been described in humans and many other organisms [2]. Among these peptides, the most well characterized are the short linear peptides (less than 40 amino acids in length) that do not contain cysteine residues. A characteristic shared by virtually all of these peptides is the presence of an amphipathic α -helical structure, which stabilizes in environments of hydrophobic nature [8] (although this helical structure has been shown not to be necessary for membrane lysis produced by a truncated form of pardaxin, an antibacterial peptide from the sole *Pardachirus marmoratus* [9]). Another characteristic shared by some of these peptides is selectivity, in that membranes from bacteria are targeted by these peptides more efficiently than mammalian plasma membranes. This selectivity is based on the complementary charge between the peptides, which are characteristically positively charged, and the negatively charged membranes of bacteria [2,3].

Structurally, these peptides typically adopt an unfolded conformation in aqueous solution. On contact with a membrane with a complementary charge, these peptides anchor to the membrane and assume an α -helical conformation. In that conformation, these peptides would either lie over the mem-

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Abbreviations: APAP, approach for detecting PAPs; PAPs, potential apoptotic peptides; SP, substance P; IP, isoelectric point; M, average helical hydrophobic moment; TI, therapeutic index

brane surface in a carpet-like arrangement (in which the peptide backbone lies parallel to the membrane), or penetrate it according to the barrel-stave mechanism (in which the peptide backbone lies perpendicular to the membrane) [2]. In either case, the integrity of the membrane would be disturbed, eventually leading to membrane lysis.

In order to optimize the homing pro-apoptotic peptide approach to cancer chemotherapy by maximizing the TI (see Section 2), we have developed a theoretical approach intended to model the properties of the antibacterial peptides that present selectivity for bacteria (and thus have very low toxic effects on mammalian cells when applied extracellularly). It is our goal in this work to develop a sequence-pattern recognition approach to detect peptides that will be toxic towards mitochondria but not to mammalian cells when applied extracellularly. We refer to the peptides identified by this approach as potential apoptotic peptides (PAPs), since they may induce apoptosis by swelling mitochondria when targeted intracellularly, as previously described [1]. We refer to the approach as APAP, as an abbreviation for approach for detecting PAPs. Using APAP, we searched the SwissProt database for PAPs and among other peptides we found that substance P (SP), an extensively studied neuropeptide present in mammals, birds and fish, has all the sequence characteristics of the PAPs. Furthermore, we found that SP is capable of swelling mitochondria and inducing the cleavage of caspase-3 zymogen, a known substrate of the active form of caspase-9 in vitro. As expected, SP demonstrated very low toxicity for eukaryotic cells when applied extracellularly, in addition to displaying toxicity towards bacterial cells. These results support our sequence-pattern recognition approach to identifying new PAPs, and suggest a new role for SP in the brain.

2. Materials and methods

2.1. Sequence-pattern recognition approach

We noticed that the known antibiotic peptides fit a pattern, which includes a low likelihood of helicity in aqueous solution, a high likelihood of helicity in the presence of negatively charged membranes, and a high isoelectric point (IP). We therefore calculated the helical probability of monomeric peptides in aqueous solution (AGADIR score), the IP and the hydrophobic moment to account for the characteristics of antibacterial peptides with low toxic activity against mammalian cells. We hypothesized that these characteristics are important in determining the selectivity observed in these peptides towards bacterial membranes and bacterial-like membranes (i.e. mitochondrial membranes).

A subset of 30 antibacterial peptides previously reported in the literature was used for calculations of AGADIR scores (A) [10], IP, and average helical hydrophobic moments (M) [11], (Tables 2A and 2B). The peptide sequences of this subset are shown in Table 1.

The TI of a peptide is here defined as the ratio between the inhibitory concentration observed with mammalian cells and the inhibitory concentration observed with bacterial cells (Tables 2A and 2B). The higher the value of this ratio is, the more specific the peptide is for prokaryotic (negatively charged) membranes.

PAPs were searched for in the SwissProt database, release 38 [12], which contains a total of 80 000 protein sequences. First, all of the peptide sequences of 40 or fewer amino acids in length were extracted from this database. Then all of these sequences (2473 database entries) were used to calculate their corresponding M , IP and AGADIR scores. Protein fragments, as opposed to peptides, were not considered in this study.

2.2. Computational resources

The PEPPLOT and ISOELECTRIC programs from the GCG package (Wisconsin package version 10, USA) were used to calculate M and IP, respectively. We averaged the non-zero α -values calculated

by the PEPPLOT program (see Section 2) for windows of eight residues. To calculate the AGADIR score, we used the AGADIR program, which was kindly provided by Dr. Luis Serrano at EMBL. The hydrophobicity of peptide sequences was obtained by calculating the average hydrophobicity of the sequence using the consensus scale reported by Eisenberg [11]. All these programs were run on a SGI Origin 2000 server.

2.3. Caspase-3 activation in a cell-free apoptosis system induced by SP

2.3.1. Preparation of cytoplasmic extracts. Cytoplasmic extracts were prepared as described before [18]. Briefly, non-apoptotic neuronal cells were sonicated and centrifuged at 16 000 $\times g$. This extract was made free of nuclei, mitochondria and did not self-prime.

2.3.2. Preparation of mitochondria. Rat and mouse liver mitochondria were prepared as described by Hovius et al., [13], with modifications as described previously [14]. Cultured cell mitochondria were prepared as described previously [15].

2.4. Protein electrophoresis and Western blots

Electrophoresis of proteins was carried out using either 8 or 12% SDS-polyacrylamide gels. Equal amounts of total protein were loaded per lane, and the proteins were separated at 4°C at 50 V through the stacking gel, and 90 V through the separating gel.

Western blot transfer of the proteins separated by electrophoresis was carried out at 4°C using PVDF membranes (0.2 mm) (Bio-Rad), at either 200 mA for 2 h. Blots were then blocked for 1 h in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween) containing 5% non-fat dried milk. Finally, the membranes were probed with an appropriate dilution (1:500 to 1:2000) of primary antibody in TBST containing 5% non-fat dried milk for 1–4 h, depending upon the antibody.

Anti-caspase-3 antibodies from mouse, rabbit and goat were purchased from Transduction Laboratories, Inc., Upstate Biotechnology, Inc. and Santa Cruz Biotechnology, Inc., respectively.

The blots were washed three times for 1 h with TBST, followed by incubation in a horseradish-peroxidase-coupled secondary antibody for 1 h in TBST containing 5% non-fat dried milk. The mouse, human, and rabbit horseradish-peroxidase-coupled secondary antibodies were from Amersham. Enhanced chemiluminescence detection of the proteins was carried out using Hyperfilm ECL (Amersham), and with Pierce SuperSignal Substrate Western Blotting reagents, or Amersham ECL reagents.

2.5. Mitochondrial swelling assays

Rat liver mitochondria were prepared as described above. The peptide concentrations used to swell mitochondria were 50 μM L-SP, 10 μM D-(LSLARLATARLAI) (negative control), or 200 μM Ca²⁺ (positive control). The swelling was quantified by measuring the optical absorbance at 540 nm.

2.6. Activity of SP on fibroblasts

10⁴ human embryonic kidney 293 cells per well were seeded into a 96-well plate. After 20 h, different aqueous dilutions (Fig. 2B) of SP (Sigma, USA), C31 and a peptide used as a control were added to the culture and the cell death was quantified by trypan blue exclusion 48 h later.

2.7. Toxicity of SP for bacterial cells

DH5α *Escherichia coli* cells were grown overnight as a pre-inoculum for the bacterial culture used in this assay. When the cells were at the end of their log phase (optical density at 600 nm of 0.8–1.0), 1 μl was used to inoculate 5 ml. Such dilution produced initial concentrations of bacteria capable of forming 10⁵–10⁶ colonies per ml in LB plates at 37°C, that is 10⁵–10⁶ colony forming units. All the bacterial cultures used in these experiments were grown in LB at 37°C. The concentration of SP required to inhibit the cell growth by 60% was determined by following bacterial growth in LB liquid in the presence of varying concentration of the peptide: 0, 1, 10, 20, 50, 125, and 250 μM. Sterilized 96-well plates of polystyrene with flat bottom and low evaporation lid (Costar, USA) were used, in a final volume of 100 μl: 50 μl of LB containing 10⁵–10⁶ colony forming units, and 50 μl of LB with a 2-fold dilution of the peptide. A 10 mM stock solution of the peptide was prepared with 5 mg of SP in 371 μl of water. Inhibition of growth was detected by measuring optical density at 600 nm with a microplate spectrophotometer SPECTRAmax (Molecular Devices,

USA) at varying times: 0, 3, 5, 6, 7 and 8 h. Each IC₅₀ was determined from at least two independent experiments performed in triplicate. Additionally, the colonies formed from each experiment were counted in LB plates at 0 and 8 h of growth.

3. Results

The antibacterial peptides analyzed and biophysical properties previously determined are presented in Tables 1 and 2A, respectively.

In order to reproduce these biophysical properties, we calculated three scores from the sequences of these peptides. Table 2A shows a subset of selected antibacterial peptide sequences (see Section 2) and the corresponding experimental values for helix formation in water and in hydrophobic environments, antibacterial activity and cytotoxic activities against mammalian cells. Table 2B shows the corresponding calculated values for *M*, IP, *A* and the TI. We observed that the antibacterial peptides presented in Table 1 are more potent against G(−) (MIC = 17.3 µg/ml on average) bacteria than to G(+) (MIC = 44.3 µg/ml), and we used the G(−) values as a reference for the TI.

Peptide sequences with values ranging from 0.4 < *M* < 0.6, *A* < 10.0 and 10.8 < IP < 11.7, were found to have the highest TI (highest specificity for bacteria) (Table 2B). These parameters were therefore hypothesized to be the signature of the PAPs. Searching for PAPs in the SwissProt database led us to identify 14 PAPs (Table 3). Two of these peptides have previously been characterized with respect to their toxicity against bacteria and mammalian cells, and in both cases a greater toxicity towards bacterial cells was observed (Table 3).

3.1. Swelling of mitochondria and activation of caspase-3 by SP

One of the PAPs identified, SP, was tested for its ability to swell mitochondria and induce caspase-3 activation in a cell-free system. This system was developed previously in our group to simulate neuronal apoptosis (see Section 2 and [14]). We observed that SP induces the swelling of mitochondria at 50 µM in our system (data not shown). At such concentration, SP was capable of releasing cytochrome *c* from mitochondria and activating caspase-3 (Fig. 1). In contrast, a peptide chosen as negative control (see Section 2) which did not present the properties of PAPs (data not shown) did not display any observable effect on mitochondria (Fig. 1).

3.2. TI of SP

The toxicity of SP against bacteria was tested and compared to the effect of SP on fibroblasts when applied extracellularly. SP was able to reduce the growth of *E. coli* cells with an IC₅₀ of 10 µM (Fig. 2B). By comparison, the negative control peptide did not have any toxicity against bacteria. In contrast, Fig. 2A shows that SP did not affect the growth of fibroblasts when applied extracellularly even at a concentration of 1 mM. These results indicate that SP has a TI > 100. Additionally, a peptide from the protein APP (the last 31 amino acids in APP, referred as C31) known to induce apoptosis when expressed intracellularly [16] was tested for its toxicity against bacteria and mammalian cells. This peptide did not present the properties (IP, *M*, *A* scores) of PAPs (data not shown). C31 did not present any observable toxicity against bacterial or mammalian cells when applied extracellularly (Fig. 2A,B).

Table 1
Peptide sequences of a subset of antibacterial peptides

Peptide name	Peptide sequence
(KIAKKIA)2NH2	KIAKKIAKIAKKIA-NH2
(KIAKKIA)3NH2	KIAKKIAKIAKKIAKIAKKIA-NH2
(KIAKLAK)2NH2	KIAKLAKKIAKLA-KLAK-NH2
(KIAKLAK)3NH2	KIAKLAKKIAKLAKKIAKLA-KLAK-NH2
(KALKALK)3NH2	KALKALKKALKKALKKALK-NH2
(KLGKKLKG)3NH2	KLGKKLKGKLGKLGKLGKLG-NH2
CecropinA	KWKLFKKIEKVVGQNIIRDGIKAGPAVAVVGQATQIAK-NH2
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH2
Magainin 2	GIGKFLHSACKFGKAFVGEIMNS-NH2
CA(1–13)M(1–13)NH2	KWKLFKKIEVKVGQGIGAVLKVLTTGLPALIS-NH2
CA(1–8)M(1–18)NH2	KWLFLKIGIGAVLKVLTTGLPALIS-NH2
Kla1	KLALKLALKWAALKLA-NH2
Kla2	KLALKALKWAALKAAKLA-NH2
Kla3	KLALKAAKALKWAALKAA-NH2
Kla7	KAIAKSILKWIKSIKAI-NH2
Kla8	KALAALLKKWAKLLAALK-NH2
Kla9	KLLAKAALKWLLKALKAA-NH2
Kla10	KALKKLLAKWLLAAAKALL-NH2
Kla11	KITLKLAIKAWKLALKAA-NH2
Kla12	KALAKALAKLWKALAKAA-NH2
m2a	GIGKFLHSACKFGKAFVGEIMNS-NH2
W16-m2a	GIGKFLHSACKFGKAWVGEGIMNS-NH2
L2R11A20-m2a	GLGKFLHSACKRGKAFVGEAMNS-NH2
I6L15-m2a	GIGKFIHSACKFGKLFVGEIMNS-NH2
I6A8L15I17-m2a	GIGKFIHAAKKEFGKLFFIGEIMNS-NH2
I6R11R14W16-m2a	GIGKFIHSACKRGFRAWVGEGIMNS-NH2
I6V9W12T15I17-m2a	GIGKFIHSVKKWGKTFIGEIMNS-NH2
100-m2a	GIAKFGKAAAHFGKKWVGELMNS-NH2
140-m2a	GIGKFLHTLKTFGKKWVGEGIMNS-NH2
160-m2a	GIGHFLHKVKSFGKSWIGEIMNS-NH2

The amino acids in the peptide sequence are represented in a one-letter code.

Table 2A
Observed characteristics of a subset of antibacterial peptides

Peptide	CD Water	Observed lipid	Antibacterial Gram(–)	Activity Gram(+)	Cytotoxicity	Reference
(KLAKKLA)2NH2	< 5	24	6	6	> 272	[8]
(KLAKKLA)3NH2	< 5	79	4	4	> 11	[8]
(KLAKLAK)2NH2	< 5	37	6	6	> 517	[8]
(KLAKLAK)3NH2	< 5	79	4	4	> 9	[8]
(KALKALK)3NH2	< 5	67	4	8	11	[8]
(KLGKKLG)3NH2	< 5	33	4	4	> 393	[8]
Cecropin A	0	75	0.2	> 300	> 200	[24]
Melittin	0	75	0.8	> 0.2	> 400	[24]
Magainin 2	0	44	4	300	300	[24]
CA(1–13)M(1–13)NH2	0	55	0.5	2	> 200	[24]
CA(1–8)M(1–18)NH2	0	63	0.3	1	> 600	[24]
Kla1	ND	73	5.2	2.6	11	[25]
Kla2	ND	68	11	45	107	[25]
Kla3	ND	59	91	> 91	> 200	[25]
Kla7	ND	70	5.6	1.4	1.8	[25]
Kla8	ND	62	5.8	3	2.5	[25]
Kla9	ND	55	6.2	1.6	1.7	[25]
Kla10	ND	62	6.1	1.5	2	[25]
Kla11	ND	69	5.3	5.3	10	[25]
Kla12	ND	67	6	1.5	10	[25]
m2a	ND	57	40	> 80	428	[25]
w16-m2a	ND	57	40	> 80	509	[25]
l2r11a20m2a	ND	45	75	> 75	> 100	[25]
i6l15-m2a	ND	57	38	38	260	[25]
i6a8l15i17m2a	ND	61	2.4	9.6	32	[25]
i6r11r14w16m2a	ND	52	37.5	> 75	303	[25]
i6v9w12t15i17-m2a	ND	64	2.3	18	56	[25]
100-m2a	ND	48	75	> 75	700	[25]
140-m2a	ND	75	13	13	35	[25]
160-m2a	ND	54	19	76	82	[25]

Peptide: see Table 1 for the amino acid composition for each peptide described in this table. CD Observed in water or lipid: percent of α -helical secondary structure determined by circular dichroism. Antibacterial activity G(+) or G(–): the minimal inhibitory concentration ($\mu\text{g/ml}$) for each peptide against Gram(+) and Gram(–) bacterial cells. Cytotoxicity: the concentration ($\mu\text{g/ml}$) required for inhibiting the growth of mammalian cells, usually red blood cells or fibroblasts.

4. Discussion

In order to optimize our homing pro-apoptotic approach to target and kill angiogenic endothelial cells supplying cancer cells, we have developed APAP, an approach to detect PAPs. APAP was originally developed to overcome the problems of toxicity and synthesis associated with our chemotherapeutic approach [1]. Positively charged PAPs, which are non-toxic outside the cell, are targeted to tumor vasculature by a fusion with a peptide that recognizes a receptor on the cell surface [17,18] and consequently internalized where they disrupt the negatively charged mitochondria, thereby exerting their pro-apoptotic effect.

We calculated the amphipathicity, IP and AGADIR scores for a subset of 30 different antibacterial peptides. The values grouping those peptides with the highest TI were considered the signature of PAPs. We searched for PAPs that resemble antibacterial ones, based on the posited relationship between mitochondrial-dependent apoptosis mechanisms and antibacterial activity. APAP provided us with a tool to identify PAPs independently of any sequence similarity with other known antibacterial or pro-apoptotic peptides. Additionally, APAP allowed us to search sequence databases systematically.

We calculated the amphipathicity and IP because amphipathic peptides are known to be membrane-associated [11], and the selectivity for recognizing bacterial-like membranes depends on the composition of the membranes [2,3]. Additionally, it has been previously recognized that hydrophobic peptides display both antibacterial activity and toxicity

against mammalian cells [19] (i.e. non-selective toxicity), thus PAPs would be expected not to be simply highly hydrophobic peptides. We observed that in our group of peptides (Table 1), all of the peptides but one were hydrophilic, constituting an appropriate group of peptides from which to select PAPs. It has been shown previously that antibacterial peptides with lower hydrophobicity display higher specificity towards Gram-negative bacteria [20]. In agreement with this notion, all the peptides analyzed in our study presented higher specificity towards G(–) bacteria as expressed by the TI values (Tables 2A and 2B).

Alternatively, the propensity to form soluble structures in water (expressed by the propensity to form secondary struc-

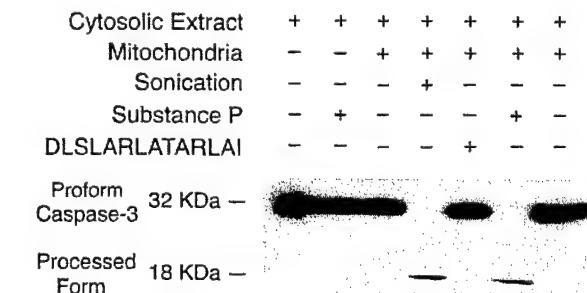


Fig. 1. Pro-apoptotic activity of SP. The release of cytochrome *c* from mitochondria and the processing of caspase-3 into the active form are shown for SP and controls (sonication, the detergent Triton X-100 and a non-toxic peptide DLSLARLATARLAI).

Table 2B
Calculated characteristics of a subset of antibacterial peptides

Peptide	A	M	IP	$\langle H \rangle$	TI
(KLAKKLA)2NH2	4.5	0.48	11.5	-0.249	45.3
(KLAKKLA)3NH2	16.2	0.48	11.7	-0.249	2.8
(KLAKLAK)2NH2	5.1	0.48	11.5	-0.249	86.2
(KLAKLAK)3NH2	17.2	0.48	11.7	-0.249	2.3
(KALKALK)3NH2	16.6	0.48	11.7	-0.249	2.8
(KLGKKLG)3NH2	1.1	0.49	11.7	-0.274	98.3
Cecropin A	1.2	0.44	11.2	-0.123	1000.0
Melittin	3.1	0.46	12.6	-0.83	500.0
Magainin 2	0.8	0.56	10.8	-0.036	75.0
CA(1–13)M(1–13)NH2	1.1	0.53	11.1	-0.46	400
CA(1–8)M(1–18)NH2	1.3	0.43	11.4	0.065	2000
Kla1	13.4	0.16	11.4	-0.025	2.1
Kla2	10.6	0.30	11.4	-0.056	9.7
Kla3	7.2	0.17	11.4	-0.087	2.2
Kla7	2.4	0.53	11.4	-0.026	0.3
Kla8	49	0.51	11.4	-0.025	0.4
Kla9	18	0.38	11.4	-0.025	0.3
Kla10	23.5	0.45	11.4	-0.025	0.3
Kla11	14.8	0.16	11.4	-0.027	1.9
Kla12	19.5	0.49	11.4	-0.056	1.7
m2a	0.8	0.56	10.8	-0.036	10.7
w16-m2a	0.9	0.49	10.8	-0.046	12.7
i2r11a20-m2a	0.9	0.51	11.1	-0.094	13.3
i6l15-m2a	0.6	0.54	10.8	-0.095	6.8
i6a8l15i17-m2a	1.1	0.55	10.8	0.016	13.3
i6r11r14w16-m2a	0.8	0.48	11.7	-0.095	8.1
i6v9w12i15i17-m2a	0.7	0.56	10.8	-0.035	24.3
100-m2a	1.1	0.46	10.8	-0.045	9.3
140-m2a	0.9	0.57	10.8	-0.049	2.7
160-m2a	0.7	0.57	10.5	-0.017	4.3

Peptide: see Table 1 for the amino acid composition for each peptide described in this table. A: AGADIR score. M: average helical hydrophobic moment. IP: estimated isoelectric point. $\langle H \rangle$: averaged hydrophobicity. TI: calculated therapeutic index.

tures in water, AGADIR score) was used in our approach. Since hydrophobicity and the propensity to form soluble structures in water are inversely related, it is expected that hydrophobic sequences will display a low AGADIR score. The inverse is not necessarily true, though; that is, peptide sequences with low AGADIR scores are not necessarily hydrophobic. Interestingly, PAPs tend to be hydrophilic with low AGADIR scores (Tables 2B and 3).

The peptides used to define the parameters of the PAPs (Table 1) are mostly synthetic peptides, with the exception of three natural peptides (magainin, cecropin A and melittin). None of these three natural peptides in Table 1 were detected

in our analysis because they were deposited in the SwissProt database in their mature form. In this form, they were longer than the cut-off value used to define the peptide database analyzed in this study (see Section 2 for a description of the peptide database used in this study). Alternatively, two cecropins (cec4_bommo, cecb_antpe) and two other natural antibacterial peptides (crbl_vescr, dms3_physa) were found in our search. In agreement with our predictions, these antibacterial peptides have been reported to have TIs similar to PAPs (Table 3). As further evidence of the validity of our approach, we tested two peptides, C31 and a control, that did not match the IP, M and A scores of PAPs (Fig. 2). The C31 peptide has

Table 3
PAPs in the SwissProt database

SwissProt name	A	M	IP	$\langle H \rangle$	Length	Antibacterial activity Gram(–)	Cytotoxicity	Reference
Boll_megpe	7.9	0.52	11.1	0.058	17			
Cec4_bommo	0.5	0.44	11.3	-0.097	35			
Cecb_antpe	0.5	0.43	11.5	-0.132	35			
Crbl_vescr	0.7	0.50	11.6	0.144	13	15	> 120	[24]
Dms3_physa	1.7	0.44	11.1	-0.024	30	2.5	80	[26]
Grar_ranru	0.04	0.53	11.6	-0.084	12			
Ranr_ranru	1.3	0.44	11.6	-0.239	17			
Npf_arttr	4.3	0.45	10.9	-0.297	36			
sp5m_bacsu	2.5	0.55	11.4	-0.095	26			
Stp_bp14	1.7	0.43	11.1	-0.278	26			
Tkna_gadmo	0.03	0.48	11.6	-0.190	11			
Tkna_horse	0.03	0.51	11.6	-0.201	11			
Tkna_oncmy	0.01	0.49	11.6	-0.175	11			
Tkna_scyca	0.03	0.49	11.6	-0.124	11			

SwissProt name: the accession name in the SwissProt database for that particular peptide. A: AGADIR score. M: average helical hydrophobic moment. IP: calculated isoelectric point. $\langle H \rangle$: averaged hydrophobicity. Cytotoxicity: the concentration ($\mu\text{g}/\text{ml}$) required for inhibiting the growth of mammalian cells, usually red blood cells or fibroblasts.

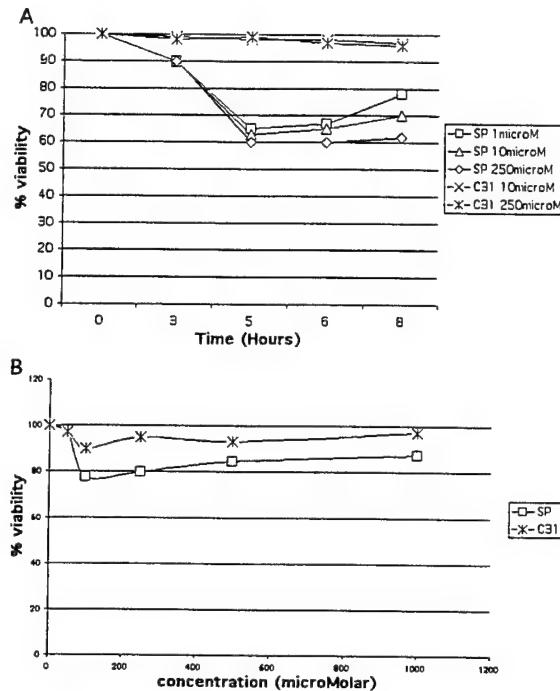


Fig. 2. Selective toxicity of SP on bacteria. The effect of SP and C31 on cell viability was measured on fibroblast cells (A) and bacteria cells (B). The viability is reported relative to a control (peptide DLSLARLATARLAI).

been shown to induce apoptosis by an unknown mechanism [16], so we considered it an interesting target for our study since we might provide some hints on the mechanism of action of C31 in addition to testing our approach. We found that none of these peptides is toxic to bacterial or mammalian cells when applied extracellularly thus confirming our predictions. Based on these results we propose that C31 may induce apoptosis by a different mechanism than PAPs.

In total, 14 sequences were identified as PAPs in the Swiss-Prot database (see Section 2). These 14 peptides can be placed into four different groups based on their known function; i.e. antibacterial peptides, neuropeptides, mast cell degranulating peptides and protein–protein interacting peptides. Two out of these four groups, antibacterial peptides and neuropeptides, represent more than 80% of the total (Table 3). Neuropeptides appear to be over-represented since there were only 48 neuropeptides in the original pool of 2473 peptides in the SwissProt database.

The special need for antibacterial peptides in the mammalian brain has been pointed out previously [21], since these may represent a more immediate line of control for bacterial infection than the immune system (which has a restricted access to the brain). Considering the properties of PAPs, our findings suggest that some previously identified neuropeptides may have antibacterial activity.

Among the neuropeptides identified as PAPs (Table 3), four were homologs of SP: tkna_gadmo, tkna_horse, tkna_oncmy, and tkna_scyc. SP belongs to the tachykinin family. Tachykinins are synthesized as larger protein precursors (usually more than 40 amino acids in length) that are enzymatically converted to their mature forms [22]. In our original search, we were able to detect only those recorded in the SwissProt

database in the active form. Analyzing all of the tachykinins deposited in the database (precursors and active forms), we found that 10 out of 61 were predicted to be PAPs (data not shown). Notably, these 10 were SP peptides from different species.

SP is known to form an α -helical structure in hydrophobic environments but not in aqueous solution [23], while it has a positive charge distribution over its sequence, supporting the finding that SP is a PAP. Therefore, the neuropeptide SP was tested for its preference for mitochondria-like membranes. The results presented in this work support our predictions that SP is a PAP. However, we did not observe a complete inhibition of *E. coli* growth, probably because of its well known short half-life in solution (minutes), while our experiments lasted for 8 h. Another possibility is that SP only displays a bacteriostatic activity, since the toxicity displayed by SP on bacterial cells was not markedly affected by the concentration of SP, as in the case of antibacterial peptides.

In developing APAP we focused on the characteristics that define selectivity rather than efficiency to kill bacteria. Therefore, it is not surprising that SP demonstrated bacteriostatic, but not bactericidal, activity. It is noteworthy that SP and most of the antibacterial peptides analyzed in this study (Table 1) are active in the low micromolar concentration range, and that SP is only 11 amino acids long. However, SP was toxic at higher concentrations than the antibacterial peptides in Table 1. We are currently working to use APAP to design more effective antibacterial peptides with higher TI values.

In conclusion, we have described a computational approach, APAP, to identify PAPs. These peptides display selectivity towards bacteria and mitochondria, with little toxic effect on eukaryotic cells when applied extracellularly, thus providing the basis for a new generation of drugs that can be present in the body without toxic effect unless they are taken in by targeted cells as we have shown previously [1]. From a public database, the approach detected mostly antibacterial peptides and neuropeptides suggesting that these neuropeptides may be the first reported with antibacterial activity. In agreement with this idea, we reported that SP is a PAP with a $TI > 100$. We speculate that these activities have been present in SP during the course of evolution of the tachykinins, which would support the possibility of a biological significance for these findings. APAP provides a method to detect and ultimately improve pro-apoptotic peptides for chemotherapy.

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Targeting the prostate for destruction through a vascular address

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Organ specific drug targeting was explored in mice as a possible alternative to surgery to treat prostate diseases. Peptides that specifically recognize the vasculature in the prostate were identified from phage-displayed peptide libraries by selecting for phage capable of homing into the prostate after an i.v. injection. One of the phage selected in this manner homed to the prostate 10–15 times more than to other organs. Unselected phage did not show this preference. The phage bound also to vasculature in the human prostate. The peptide displayed by the prostate-homing phage, SMSIARL (single letter code), was synthesized and shown to inhibit the homing of the phage when co-injected into mice with the phage. Systemic treatment of mice with a chimeric peptide consisting of the SMSIARL homing peptide, linked to a proapoptotic peptide that disrupts mitochondrial membranes, caused tissue destruction in the prostate, but not in other organs. The chimeric peptide delayed the development of the cancers in prostate cancer-prone transgenic mice (TRAMP mice). These results suggest that it may be possible to develop an alternative to surgical prostate resection and that such a treatment may also reduce future cancer risk.

Diseases affecting the prostate have gained major significance clinically and economically, primarily because of the increasing average age of the male population in the industrialized countries. Benign prostate hyperplasia affects to some degree most elderly men. Even more serious, the prostate is a frequent site of cancer. Some autopsy studies find that most men older than 70 have occult or overt cancer in the prostate (1). The surgical therapies of prostate hypertrophy and prostate cancer are associated with serious side effects, such as incontinence and impotence.

We have sought to develop a strategy that would provide a less traumatic treatment for prostate disease than is currently available. Our strategy is based on identification of peptides that home to specific sites in the vasculature by *in vivo* screening of intravenously injected phage libraries. These studies have revealed a surprising degree of specialization in the endothelia of various normal tissues (2, 3). Screening phage libraries for tumor homing has yielded a collection of peptides that home to tumor vasculature (4). We and others have used these tumor-homing peptides to direct therapies into tumors in mice (4, 5). We report here the identification of peptides that home to the vasculature of the prostate and the use of one of these homing peptides to deliver a proapoptotic peptide to the prostate.

Materials and Methods

Materials. Peptides were synthesized to our specifications by AnaSpec (San Jose, CA) or by our Peptide Synthesis Facility. The peptides were purified by HPLC and their identity was confirmed with mass spectrometry.

Apotag Kit for TUNEL staining was purchased from Intergen (Purchase, NY). Testosterone pellets (12.5 mg) and control pellets were from Innovative Research of America (Sarasota, FL), and controlled release pumps from Alzet (Mountain View,

CA). The pumps were loaded with peptides following the manufacturer's instructions.

Mice. CD-1 male mice (The Jackson Laboratories) were used for phage screening at an age of 2–4 months. Transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, kindly provided by Norman Greenberg, Baylor College of Medicine, Houston) were bred at our Animal Facility.

Phage Libraries and Library Screening. The phage libraries were prepared in the fUSE5 vector as described (6, 7). The primary library contains about 5×10^9 individual recombinant phage. For the library screening, CD-1 mice were anesthetized with Avertin (0.015 ml/g) and injected intravenously (tail vein) with phage libraries containing 10^9 transducing units diluted in 200 μ l of DMEM. The phage was rescued from tissues by bacterial infection (2), and about 300 individual colonies were grown separately. The bacterial cultures were then pooled and the amplified phage were injected into mice as described above. To test individual phage for homing, 10^9 colony-forming units (cfu) (fUSE5) or 10^{10} plaque-forming units (pfu) (T7), diluted in 200 μ l of PBS, were injected. The SMSIARL insert and its scrambled variant were cloned to the T7 phage (T7select415-1 vector; Novagen), and the resulting phage was tested as described (8).

Results

In vivo screening of a fUSE5 phage heptapeptide library for prostate-homing peptides (6) yielded two phage that accumulated selectively in the prostate. One of these phage, displaying the peptide SMSIARL (single letter code), homed to the prostate 15 times more than nonrecombinant control phage (Fig. 1a). The other prostate-selected phage (VSFLEYR) gave a prostate-homing ratio of ≈ 10 . The homing of the SMSIARL phage to prostate tissue was inhibited when synthetic SMSIARL peptide was injected together with the phage, but not when an unrelated peptide was injected (Fig. 1a). The SMSIARL phage

Abbreviation: TRAMP, transgenic adenocarcinoma of the mouse prostate.

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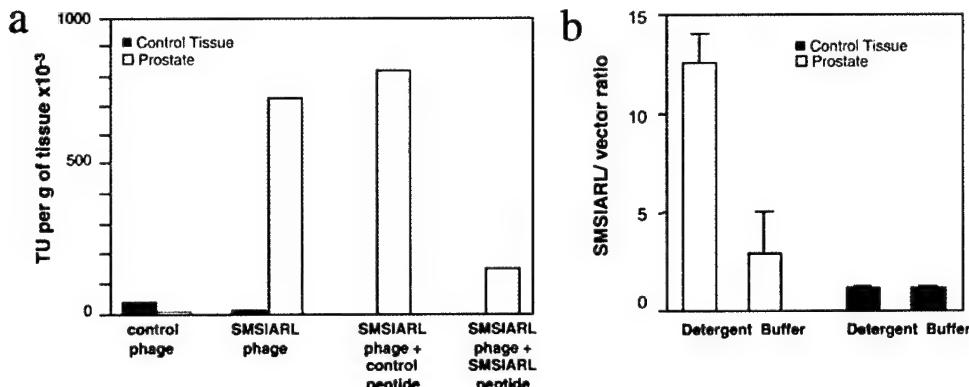


Fig. 1. Specific homing of phage to the prostate. (a) Phage selected for prostate homing accumulates specifically in the prostate and the homing is inhibited by soluble peptide. The SMSIARL fUSE5 phage, identified by *in vivo* screening, was tested for prostate homing. This phage and an irrelevant control phage were injected intravenously to male mice [10⁹ colony forming units (cfu) per mouse] and the phage were rescued from various tissues based on their ability to infect a host bacteria. As indicated, 200 µg of the SMSIARL peptide or a control peptide (CARAC) was included in the injection to test inhibition of SMSIARL phage homing. (b) The SMSIARL peptide directs specific homing of T7 phage to the prostate. The SMSIARL sequence was cloned to the coat protein of the T7. A 1:10 mixture of SMSIARL and nonrecombinant control T7 phage [10¹⁰ plaque-forming units (pfu)] was injected and allowed to circulate for 7 min. Phage was extracted from prostate and brain with buffer (PBS), or a detergent solution (0.5% Nonidet P-40 in PBS) and plated, and 32 colonies were randomly chosen for PCR. The PCR products of SMSIARL and control phage DNA were distinguished on the basis of a size difference in a 4% agarose gel. (Control tissue was brain.)

homed also to the rat prostate tissue (not shown). The SMSIARL peptide when cloned into the T7 phage (6) showed a similar homing specificity for the prostate.

Phage displaying a scrambled variant of this peptide (LAM-SRIS) showed no homing to the prostate. The T7 SMSIARL phage was not enriched in the brain (Fig. 1b), salivary gland, kidney, testis, thymus, pancreas, skeletal muscle, or lung (not shown). We also confirmed the homing specificity by co-injecting

SMSIARL phage and nonrecombinant phage; the ratio of the two types of phage in the prostate was determined by PCR. The SMSIARL phage homed to the prostate 10–15 times more than the nonrecombinant phage. The recovery of the SMSIARL phage was more than 5-fold higher when the tissue was extracted with detergent rather than buffer alone. The brain as a control organ showed no enrichment with or without detergent (Fig. 1b). The greater phage recovery after lysis of the tissue with deter-

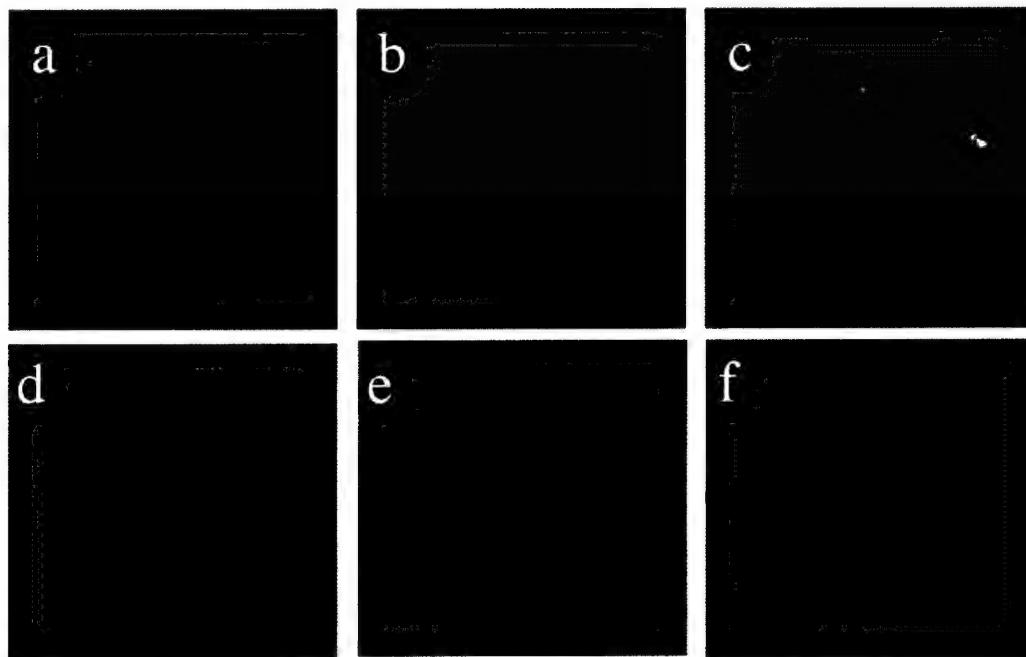


Fig. 2. Immunohistochemical staining of phage within prostate endothelial cells after i.v. injection into mice. SMSIARL-phage preparation was injected intravenously into mice. After 7 min circulation, animals were perfused with PBS, the prostate (a–c), brain (d–f), and various control organs were removed, processed for frozen sectioning, and stained with a polyclonal antibody against T7 phage (FITC; a and d) and CD31 (rhodamine; b and e). Merge with nuclear counterstain with DAPI (c and f). Control organs (kidney, spleen, lung; not shown) were negative for the phage, except for liver and spleen, where the reticuloendothelial tissue traps phage nonspecifically (4). (Magnification: ×400.)

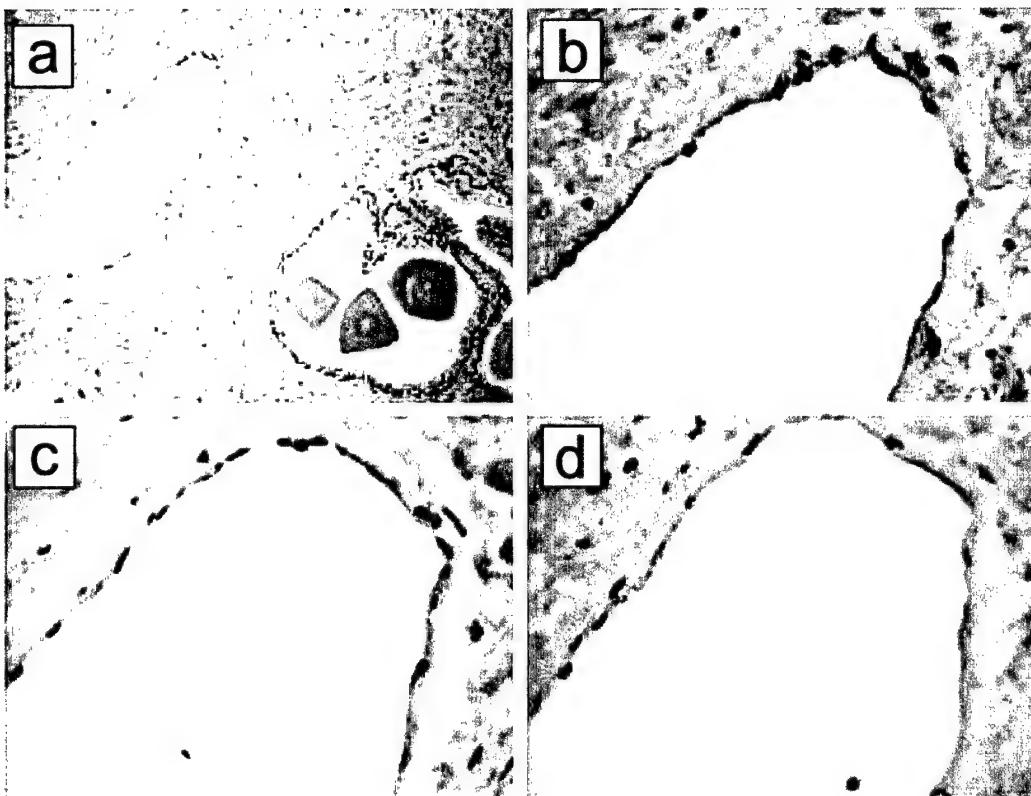


Fig. 3. SMSIARL phage binds to endothelium in human prostate. A human prostate tissue section containing both normal and cancerous tissue was overlaid with the SMSIARL phage (10^9 cfu/ml) and the binding of the phage was detected with anti-M13 phage antibody and peroxidase staining. (a) An overview ($\times 200$); (b) a detail from a at a higher magnification ($\times 400$). Staining of the endothelium is seen. (c) Overlay with phage that contains no peptide insert produces no endothelial staining. (d) The SMSIARL-phage staining is inhibited when soluble SMSIARL peptide is included in the overlay at 0.3 mg/ml.

gent suggests that the SMSIARL phage may have been taken up into cells.

Antibody staining of the phage in tissue sections from mice injected intravenously with the T7 SMSIARL phage revealed staining in the prostate 7 min after an i.v. injection (Fig. 2). The phage staining colocalized with staining for the blood vessel marker CD31, indicating homing to blood vessels in the prostate. No specific staining was seen in control organs, or in prostate or control organs of mice injected with a nonrecombinant control phage. The phage staining appeared to be intracellular, supporting the detergent extraction results shown in Fig. 1b. Overlay of tissue sections from human prostate with the SMSIARL phage indicated that this phage also binds to the endothelium of human prostate blood vessels the same way it binds to the mouse prostate vessels (Fig. 3). Significantly, vessels in hypertrophic human prostate tissue bound the SMSIARL phage. No binding of this phage was detected in the blood vessels in several other human tissues. Similar localization results were obtained with the free SMSIARL peptide coupled to fluorescein (data not shown).

We next studied the ability of the SMSIARL peptide to deliver a biologically active compound to the prostate. D(KLAKLAK)₂ is an amphipathic D-amino acid peptide that binds selectively to bacterial, but not eukaryotic cell membranes (9). It has antibacterial activity, but is relatively nontoxic to eukaryotic cells. We have previously shown that D(KLAKLAK)₂, if delivered into mammalian cells, disrupts mitochondria (mitochondrial membranes resemble those of bacteria), initiating apoptosis (10). Conjugated through a G-G linker to a homing peptide that

homes to tumor vasculature, D(KLAKLAK)₂ yields a chimeric compound that is selectively cytotoxic to angiogenic endothelial cells and has antitumor activity *in vivo* (10). We used the same strategy to prepare a proapoptotic chimera that targets the vasculature of the normal prostate, and studied its ability to cause selective tissue destruction in the prostate.

Mice were injected with 250 μ g of the targeted SMSIARL-GG-D(KLAKLAK)₂ chimeric compound and the prostates were collected after 1, 4, 8, 12, 16, 24, and 48 h, and after 7 days. Control groups received D(KLAKLAK)₂ coupled to a non-homing scrambled peptide (LAMSRIS), SMSIARL and D(KLAKLAK)₂ as an uncoupled mixture, or buffer alone. A total of 62 mice treated with SMSIARL-GG-D(KLAKLAK)₂ were evaluated. In prostates collected 16 h or later after the injection, histology revealed an unevenly distributed destruction of the prostate glandular epithelial cells that in some areas included epithelial shedding and destruction of entire glandular structures (Fig. 4a and b). These changes were still present 7 days after the treatment and no mitotic figures were observed, suggesting sustained damage and poor regeneration (not shown). Electron microscopy showed extensive destruction of intracellular organelles in the SMSIARL-GG-D(KLAKLAK)₂-treated, but not control-treated, mice (Fig. 4 c and d). Tissue damage was also evident from an increase in TUNEL-positive vascular and glandular cell nuclei in the prostates of mice treated with SMSIARL-GG-D(KLAKLAK)₂ (not shown). The prostates of control animals displayed only rare degenerating epithelial cells and all other organs examined (brain, heart, liver, kidney, lung, urothelium) were histologically normal during or after treatment with each of the compounds (Fig. 4 e–h).

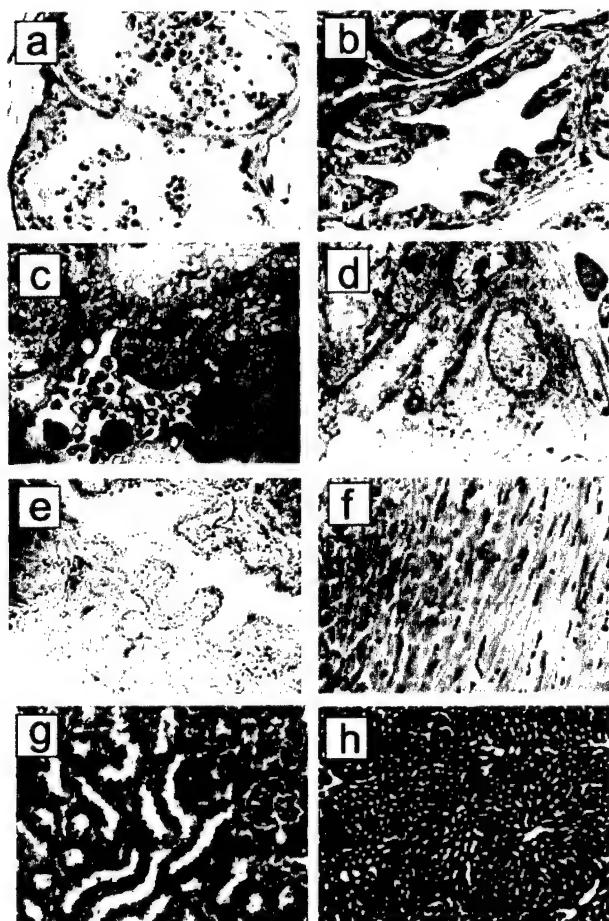


Fig. 4. Targeted proapoptotic peptide to mouse prostate vasculature causes tissue damage in prostate but not in other tissues. Mice received an i.v. 250 μ g injection of the SMSIARL-GG- D (KLAKLAK) $_2$ or an equivalent dose of SMSIARL and D (KLAKLAK) $_2$ as uncoupled peptides (control-treated mice). The mice were killed 24 h after the injection. Prostates were fixed in paraformaldehyde or glutaraldehyde solution and processed for light microscopy by staining with hematoxylin/eosin (H&E) or electron microscopy. Light microscopy showed focal loss of cell borders and epithelial shedding in the ventral lobe of prostates from the SMSIARL-GG- D (KLAKLAK) $_2$ group. (a) H&E-stained micrograph shows massive glandular destruction with nearly complete shedding of the glandular epithelial cells into the lumen. (b) A representative micrograph of normal prostate tissue from a mouse treated with the uncoupled peptide mixture. (Magnification in a and b, $\times 400$.) (c) An electron microscopic image of a single epithelial cell from a SMSIARL-GG- D (KLAKLAK) $_2$ -group prostate. The cell has sloughed off into the glandular lumen and massive destruction of its organelles is seen. (d) A representative micrograph of normal prostate shows intact cellular structure. (Magnification in c and d, $\times 6,000$.) Light microscopy shows no damage to bladder (e; $\times 200$), heart (f; $\times 400$), kidney (g; $\times 400$), or liver (h; $\times 400$).

To effect sustained levels of the compounds used in the treatments, we used an implanted peristaltic pump for controlled release. Each pump was loaded with either SMSIARL- D (KLAKLAK) $_2$ or an uncoupled mixture of SMSIARL and D (KLAKLAK) $_2$. The animals were killed after 1 week, and their organs processed for histology. In another control experiment, we also implanted s.c. testosterone pellets to eliminate any variation in the sensitivity of prostate tissue caused by possible fluctuations in endogenous androgen levels (11). Seven days later, controlled release pumps loaded with the peptides were implanted on the peritoneal area opposite the pellets.

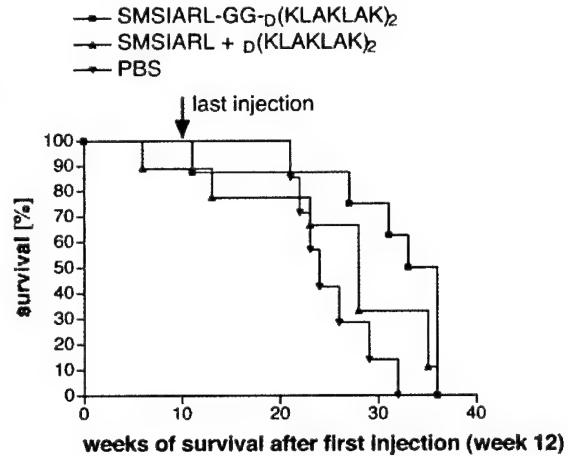


Fig. 5. Survival of TRAMP mice treated with SMSIARL-GG- D (KLAKLAK) $_2$ or control materials. The treatment was initiated at 12 weeks of age. Male mice (ten per group) received i.v. injections of SMSIARL-GG- D (KLAKLAK) $_2$ peptide (200 μ g per dose), or an equivalent dose of SMSIARL and D (KLAKLAK) $_2$ as uncoupled peptides (control-treated group). The injections were given once a week for a total of ten doses. The mice in the SMSIARL-GG- D (KLAKLAK) $_2$ group survived significantly longer than the control mice treated with the uncoupled peptide mixture or with buffer.

SMSIARL-GG- D (KLAKLAK) $_2$ consistently produced damage in the prostate (data not shown).

The tolerated dose of SMSIARLGG- D (KLAKLAK) $_2$ was limited by acute toxicity of the compound; the dose could be increased by giving the injection slowly over several minutes. Mice injected with SMSIARL-GG- D (KLAKLAK) $_2$, as well as those injected with equivalent amount of nonconjugated mixture of the homing peptide and proapoptotic peptide, showed marginal elevation of serum parameters of liver (ALT, AST, GGT) and kidney (creatinine and blood urea nitrogen) function. The levels returned to normal 1 week after the treatment. In one experiment, four mice that had been treated with four weekly injections of SMSIARL-GG- D (KLAKLAK) $_2$ were allowed to mate. Vaginal plugs showed that mating had occurred and litters were born in each case. These results suggest that SMSIARL-GG- D (KLAKLAK) $_2$ causes damage in the prostate, while other tissues are spared and the mice remain fertile.

We next analyzed the effect of a systemic SMSIARL-GG- D (KLAKLAK) $_2$ treatment on the longevity of TRAMP mice (12). Two independent experiments gave similar results; one of the experiments is shown in Fig. 5. The SMSIARL-GG- D (KLAKLAK) $_2$ survived significantly longer than the control groups that received the uncoupled peptides or buffer ($P < 0.01$ for both; Log Rank test).

Discussion

We show here that peptides selected from phage libraries for homing to the prostate vasculature reveal tissue-specific features in the blood vessels of the prostate. We also show that a peptide capable of homing to the blood vessels in the prostate can target a proapoptotic peptide to the prostate, and that systemic treatment with this targeted compound can cause destruction of prostate tissue and delay the development of prostate cancer in mice. Our results show that, like the vasculature of many other tissues analyzed in previous work (2–4), the vasculature of the prostate is biochemically distinct. The accumulation of the SMSIARL phage and fluorescein-labeled SMSIARL peptide in the prostate blood vessels after an i.v. injection indicates that this peptide binds selectively to the blood vessels in the prostate. The

selective destruction of prostate tissue caused by targeting of a proapoptotic peptide to the prostate with the SMSIARL homing peptide supports this conclusion.

The molecular nature of the vascular specialization is incompletely understood. We have identified the receptor for a peptide that homes to lung vasculature as membrane dipeptidase (13). Others have shown that a modified von Willebrand factor promoter is activated in endothelial cells in a tissue-specific manner under the influence of the surrounding parenchymal tissue (14), providing one possible regulatory mechanism for the expression of tissue-specific endothelial markers. Perhaps prostate tissue induces receptors for SMSIARL in the resident endothelium. Although the molecule the SMSIARL peptide binds to in the prostate vasculature remains to be identified, our results suggest some practical applications.

The destruction of prostate tissue by the SMSIARL-targeted proapoptotic peptide is likely to be secondary to loss of blood vessels, the main target of the homing peptide. However, we cannot exclude a direct effect on prostate epithelial cells. The tissue damage was specific for the prostate, suggesting that it may be possible to develop a "medical prostatectomy" procedure based on this principle. Such a procedure could provide an

alternative treatment for prostate hypertrophy. Furthermore, the proapoptotic peptide treatment postponed the development of prostate cancer in TRAMP mice. We attribute the effect in the TRAMP mice to a reduction in the number of target cells available for malignant transformation, because the SMSIARL peptide does not home to the vessels in the TRAMP tumors (W.H. and E.R., unpublished result). The lifespan extension in our treated TRAMP mice was 6–8 weeks, close to 20% of the lifespan, even though the treatment works against a tremendous oncogenic pressure in these transgenic mice (12, 15). In human terms, this would mean postponement of prostate cancer development for several years. A medical treatment that reduces the size of the prostate and at the same time delays the development of prostate cancer could be an extremely useful procedure.

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Anti-tumor activity of *de novo* designed protein

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We previously designed *de novo* the first native-like pore-forming protein, small globular protein (SGP), based on the colicin family of bacteriocins. Surprisingly, we show here that it has strong anti-tumor activity in a mouse model of human cancer. Furthermore, this is the first report of significant, or any, regression in human tumor xenografts by a pore-forming protein, synthetic or natural. We recently reported on a new systemic cancer therapy using novel targeted pro-apoptotic peptides that selectively induce apoptosis in tumor blood vessel endothelial cells. SGP is the prototype for an entirely new class of *de novo*-designed, pore-forming, anti-tumor proteins that can be locally applied alone or in combination with such a systemic approach. The novelty and broad significance of our finding is that the first *de novo*-designed pore-forming protein is not only biologically functional, but is also a potent cancer therapeutic.

The *de novo* design of proteins is an emerging area of research that has expanded our understanding of protein folding, and laid the groundwork for the creation of unprecedented structures with unique functions^{1,2,3}. For example, the propensity of amphipathic peptides to assemble in aqueous solution, and of the β -turn to form a loop, have been successfully employed to design coiled-coil proteins⁴⁻⁶, various helix bundle proteins⁷⁻¹², and β -structural proteins^{13,14}. The *de novo* design of proteins with true *biological* function, such as heme binding, catalysis, or the formation of a membrane pore or channel, is perhaps the most challenging goal of peptide chemistry^{3,15-21}. Although progress has been made in designing membrane proteins that are correctly incorporated into membranes, few attempts have been made to design proteins capable of forming channels or pores^{21,22}.

We previously designed and synthesized the first native-like pore-forming protein, small globular protein (SGP), based on the colicin family of bacteriocins²³⁻²⁶. SGP is a 69 amino acid, 4-helix bundle protein, composed of 3 amphipathic helices, made from Leu and Lys residues, which surround 1 hydrophobic helix made from Ala residues, creating a single hydrophobic pocket (Fig. 1a,b)^{27,28}. It is monomeric in solution, and is denatured in a highly cooperative manner, characteristic of native globular-like proteins. Indeed, it does not completely denature even at high temperature (90°C), and high Gu•HCl concentration (5M, midpoint concentration). Moreover, 1-anilinonaphthalene-8-sulfonate (ANS) binding experiments demonstrate that it adopts a native-like structure.

While most naturally occurring, pore-forming proteins maintain their tertiary structure when disrupting membranes, the colicins undergo a spontaneous transition from a native folded state in solution to an open umbrella-like state in membranes²³⁻²⁶. SGP was designed to mimic this insertion mechanism (Fig. 1c)^{27,28}. The transition was confirmed in synthetic bilayers, where SGP formed a uniform size pore (14pS)²⁷.

We recently developed a novel cancer treatment using targeted pro-apoptotic peptides that selectively destroy tumor blood vessel endothelial cells, while remaining relatively non-toxic to normal blood vessel endothelial cells²⁹. These short peptides (~20 amino acids) are composed of two functional domains — one a tumor blood vessel homing motif and the other a programmed cell death inducing sequence. The homing domain (e.g. CNGRC) is designed to guide the peptide to targeted cells and allow its internalization. The pro-apoptotic domain (e.g. KLAKKLAKLAK) is designed to be non-toxic outside of cells, but toxic when internalized into targeted cells by the disruption of mitochondrial membranes. This domain specifically disrupts negatively charged membranes, such as those found in mitochondria, but not neutral or zwitterionic membranes, such as those found in the outer leaflet of mammalian plasma membranes (excluding neurons).

Given that SGP forms pores in synthetic membranes, we asked whether or not it could disrupt biological membranes, and whether or not it would have the same *in vitro* membrane disruption specificity of targeted pro-apoptotic peptides. Moreover, we wondered if SGP could be used successfully *in vivo*, as an anti-tumor agent.

Results

SGP kills tumor cells *in vitro*.

We evaluated SGP in the same assays used to investigate the membrane disrupting properties of targeted pro-apoptotic peptides — mitochondrial swelling, a mitochondria-dependent cell-free system of apoptosis, and cytotoxicity^{29,30}. First we considered the effect of SGP on negatively charged membranes, with mitochondria as the model biological system. SGP induced marked mitochondrial swelling at a concentration of 1 μ M (Fig. 2a, purple line). Mild swelling was evident even at 500 nM (data not shown). 40 μ M of Ca²⁺ served as the positive control (Fig. 2a, blue line). The negative control used throughout this work is the non- α -helix forming

peptide DLSLARLATARLAI, and it did not induce mitochondrial swelling (Fig. 2a, green line)^{29,30}. Next we looked at the effect of SGP on negatively charged membranes in a cell-free system of apoptosis. This system was developed to evaluate peptides and proteins for their ability to disrupt mitochondrial membranes to the extent that cytochrome c (among other apoptosis inducing factors) is released from the inter-membrane space, leading to caspase-3 processing, a hallmark of apoptosis activation³⁰. Although 10 μ M SGP did not activate cytosolic extract alone (data not shown), it did activate a mitochondria-dependent cell-free apoptosis in a system composed of mitochondria suspended in cytosolic extract, as measured by the characteristic caspase-3 processing from an inactive zymogen to active protease (Fig. 2b, lane 5)²⁹⁻³¹. Triton X-100, the positive control, also activated the mitochondria-dependent cell-free system (Fig. 2b, lane 4). The negative control peptide was inactive in the cell-free system (Fig. 2b, lane 6)^{29,30}. In contrast, the pro-apoptotic domain of targeted pro-apoptotic peptides induced mitochondrial swelling at 10 μ M, and activated cell-free apoptosis at 100 μ M. Thus, SGP is approximately 10 times more potent in disrupting negatively charged membranes than targeted pro-apoptotic peptides²⁹.

Finally, we considered the effect of SGP on uncharged (or lowly charged) membranes by comparing its cytotoxicity to that of targeted pro-apoptotic peptides using 3 human cell lines — the Kaposi sarcoma derived cell line KS1767, the breast carcinoma derived cell line MDA-MD-435, and the microvessel endothelial cell line DMEC^{32,33}. The treatment of KS1767 cells with >10 μ M SGP led to an extremely rapid cell death, characterized by 100% loss of viability within 60 sec (Fig. 3a). The death was characterized morphologically by the total lack of the classic signs of both necrosis, such as swelling, and apoptosis, such as nuclear fragmentation. The cells appeared to have fixed plasma and nuclear membranes, and many tiny membrane bulges were evident on these surfaces. Lowering the concentration of SGP to

values in between 10 μM and 5 μM led to the induction of necrosis (scored morphologically), resulting in almost 100% loss of viability over 60 min (Fig. 3b). Lowering the concentration of SGP even more, below 5 μM , led to the induction of apoptosis over the course of 24 hrs (Fig. 3c). Apoptosis was confirmed by caspase-3 assay. KS1767 cells are unaffected by a 24 hr incubation in 100 μM of the control peptide (Fig. 3d). However, the classic morphological signs of apoptosis, nuclear condensation (Fig. 3e, short arrow) and plasma membrane blebbing (Fig. 3e, long arrow), are apparent in KS1767 cells after a 24 hr treatment with 3 μM SGP. Similar results were obtained using the MDA-MD-435 and DMEC cell lines, although the DMECs were somewhat more resistant to killing (data not shown). This may be due to the fact that although the outer leaflet of the plasma membrane is generally electrically neutral or zwitterionic for cells of mammalian origin (except neurons), the outer leaflet for tumor cells is often negatively charged due to the presence of some phosphatidylserine³⁴. In contrast, the pro-apoptotic domain of targeted pro-apoptotic peptides did not induce cell death in KS1767, MDA-MD-435, and DMEC cells until its concentration was greater than 300 μM , 30 times higher than the concentration of SGP required to kill cells²⁹. The negative control peptide was not lethal to mammalian cells^{29,30}.

The data above demonstrate that SGP is a more potent membrane-disrupting agent than the pro-apoptotic domain of targeted pro-apoptotic peptides, but also that it is non-specific, in that it disrupts both negatively charged and neutral membranes at approximately the same concentration (~3 μM).

SGP kills tumor cells *in vivo*.

Although SGP was not specific for tumor cells, we were impressed by its potent destruction of those cells, and so we proceeded to test it in nude mice bearing human MDA-MD-435 breast carcinoma xenografts to determine if injecting it directly into

tumors would reduce their volume and retard metastasis. Our working hypothesis was that SGP would be degraded by proteolysis or sequestered within the center of tumors, making it unlikely that SGP would have any therapeutic effect.

Surprisingly, final tumor volumes were significantly smaller in the SGP treated mice than in the PBS treated mice (Fig. 4a). Starting tumor volumes ranged from standard sizes of about 100 (mm^3)³ to large sizes of about 600 (mm^3)³. The tumor-bearing mice were given four weekly treatments of PBS, or 100 μM or 1 mM SGP (40 μl /treatment given in 5 μl increments). After a subsequent 4-week lapse in treatment, the tumor volumes were measured at 8 weeks. The average final tumor volume of each SGP treated group was 5 times less than the average final tumor volume of the PBS treated group (Fig. 4a, bars). There was little, if any difference between the average final tumor volumes of the 2 SGP treatment groups. Furthermore, 2 out of 10 mice had tumors which flattened-out and seemed to disappear (Fig. 4b, middle and right arrows). These mice appeared cured, living > 4 months after tumor implantation, before being euthanized for histological/toxicological studies, suggesting that both primary tumor growth and metastasis were inhibited. Surgical examination of the tumor sites revealed no sign of the primary tumors.

We also considered whether or not SGP would produce adverse side effects such as a blister, lesion, or scar when injected under normal skin. Strikingly, in all eight mice tested, SGP did not produce any surface effect when injected sub-cutaneously (Fig. 4c) (or intra-dermally, data not shown) as compared with PBS controls. There was some typical ulceration/scabbing of both the untreated and treated tumors due to surface necrosis (Fig. 4b, arrows).

Histopathological analysis of the SGP-treated MDA-MD-435 human breast carcinoma xenografts showed widespread apoptosis (Fig. 5b), as compared with PBS treated tumors (Fig. 5a). Many condensed nuclei are apparent (Fig. 5b, short arrows), as is the total lack of effect on the extracellular matrix (Fig. 5b, long arrows).

Apoptosis was confirmed by caspase-3 assay (data not shown). It is noteworthy that 100 μ M SGP induces an almost immediate cell death *in vitro* that is neither apoptotic or necrotic, while 100 μ M SGP induced apoptosis *in vivo*. Higher SGP concentrations may be required *in vivo*, due to some proteolysis of the protein, although work is underway to evaluate lower concentrations. SGP treated human KS1767 Kaposi's sarcoma xenografts showed the same effects (Fig. 5c,d). It is important to emphasize that tumor volume measurements (Fig. 4a) underestimate the efficacy of anti-tumor agents because dead cells still occupy volume until scavenged (Fig. 5b,c). As expected for a locally applied therapeutic, histological analysis of the major organs of SGP treated mice showed no overt pathology (data not shown).

Discussion

SGP represents a new class of anti-cancer proteins that can be optimized for maximum therapeutic effect by adjusting the properties of its four helices such as residue placement, domain length, and hydrophobicity^{29,35}. Indeed, work is underway to design new membrane-specific derivatives that will fold in aqueous phase, and unfold only in the presence of negatively charged membranes, such as those of tumor cell plasma membranes^{29,30}. Even in its present form, SGP can be used alone as a local tumor treatment (e.g. melanomas, Kaposi's sarcomas), or in combination with a systemic approach, such as targeted pro-apoptotic peptides²⁹. It could also be applied as an organ ablation agent for conditions such as prostate hyperplasia³⁶.

Although SGP appears to be a non-specific membrane-disrupting agent, it is specific in the sense that the disruption is limited (Figs. 3e and 5b,d). SGP can only physically disrupt the membrane architecture, leading to cell lysis, and the creation of cellular debris on an organellar or sub-organellar scale. It cannot act like a detergent and solubilize membranes completely, down to individual phospholipid molecules, or chemically break covalent bonds, such as those holding proteins to-

gether within the extra-cellular matrix. This might explain the lack of SGP side-effect when injected sub-cutaneously or intradermally.

While this is the first report of a pore-forming peptide or protein, natural or synthetic, being applied to treat established human tumor xenografts, several pore-forming peptides and proteins have been shown to have moderate efficacy in killing tumor cells *in vitro*. For example, the anti-bacterial peptides magainin (and synthetic derivatives),³⁷ cecropin (and synthetic derivatives),³⁸ granulysin,³⁹ and NK-lysin⁴⁰ are toxic to tumor cells in culture. The pore-forming protein verotoxin 1 (a colicin) has also been shown to have a toxic effect on tumor cells *in vitro*⁴¹. Magainin, cecropin and verotoxin 1 also had some efficacy *in vivo*, in mice bearing murine tumors^{37,38,41}. The anti-tumor effects of the pro-apoptotic bax and bak genes have been evaluated in tissue culture and in nude mice bearing human tumor xenografts^{42,43}. Intratumoral injection of adenovirus vector expressing the bax and bak gene suppressed tumor growth. However, unlike SGP, it remains uncertain whether or not Bax and Bak kill by forming pores, or by interacting with other oncoproteins and/or complexes such as Bcl-2 and the permeability transition pore complex (PTPC), respectively⁴⁴.

The *de novo* design of proteins with biological function is in its infancy. SGP is one of only a handful of such proteins^{1,3}. Our work provides a glimpse at the potential of what might be achieved with them. It is remarkable and promising that the first practical application of the first *de novo* designed pore-forming protein is in the treatment of human cancer.

Methods

Reagents. SGP was synthesized according to the Fmoc procedure starting from Fmoc-Leu-PEG [poly(ethylene glycol)] resin by using a Miligen automatic peptide synthesizer (Model 9050) to monitor the deprotection of the Fmoc group by the UV absorbance²⁷. After cleavage from the resin by trifluoroacetic acid, the crude peptide obtained was purified by HPLC chromatography with an ODS column, 20 X 250 mm, with a gradient system of water/acetonitrile containing 0.1% trifluoroacetic acid. Amino acid analysis was performed after hydrolysis in 5.7 M HCl in a sealed tube at 110 °C for 24 hr. Analytical data obtained were as follows: Gly, 6.2 (6); Ala, 9.5 (10); Leu, 26.5 (25); Asp, 3.0 (3); Pro, 2.9 (3); Tyr, 3.1 (3); Lys, 18.9 (18). Molecular weight was determined by the FAB-mass spectrum using a JEOL JMX-HX100: base peak, 7555.1; calculated for C₃₆₇H₆₃₉O₇₇N₉₁•H⁺, 7554.8. Peptide concentrations were determined from the UV absorbance of Trp and three tyrosines at 280 nm in buffer ($\epsilon = 8000$). Gel filtration HPLC chromatography was performed by using Tris buffer (10 mM Tris, 150 mM NaCl, pH 5.0 or pH 7.4) on COSMOSIL 5DIOL-300 (Nakalai Tesk, Kyoto, Japan). Anti-caspase-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and N-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA; BioMol, Plymouth Meeting, PA) were commercially obtained. The negative control peptide (DLSLARLATARLAI) was synthesized to our specifications at higher than 90% purity by HPLC (AnaSpec, San Jose, CA).

Computer model. The computer generated model of SGP was made with the program Insight II (Molecular Simulations Inc., San Diego, CA) running on an Octane SSE workstation (Silicon Graphics, Cupertino, CA).

Cell culture. All cells lines were of human origin. The primary dermal microvessel endothelial cells (DMEC) were obtained commercially (CADMECs, Cell Applications, San Diego, CA), and grown in proprietary CADMEC growth media, as described previously²⁹. The Kaposi's sarcoma-derived cell line KS1767, and the breast carcinoma cell line MDA-MB-435 were also described previously²⁹⁻³¹. Both lines were cultured in 10%FBS/ DMEM containing antibiotics.

Mitochondrial swelling. Rat liver mitochondria were prepared as described³⁰. The concentrations of reagents used were 10 μM SGP, 100 μM DLSLARLATARLAI (negative con-

trol), or 40 μ M Ca⁺² (positive control). Proteins or peptides were added to mitochondria in a cuvette, and swelling was quantified by optical absorbance at 540 nm.

Cell-free apoptosis. Cell-free systems were reconstituted as described^{29,30}. Briefly, for the mitochondria-dependent reactions, rat liver mitochondria were suspended in normal (non-apoptotic) cytosolic extract of KS1767 cells. SGP was added at a concentration of 10 μ M. After incubation for 2 hrs at 30°C or 37°C, mitochondria were removed by centrifugation, and the supernatant was analyzed by SDS/PAGE immunoblotting (12% gels, Biorad, Hercules CA). Proteins were transferred to PVDF membranes (Biorad, Hercules CA) and incubated with anti-caspase-3 antibody, followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ).

Caspase activity. The caspase activity of cell lysates was measured as described^{29,30}. Briefly, aliquots of cell lysates (1 μ l lysate, 8-15 mg/ml) were added to 100 μ M DEVD-pNA (100 μ l, 100 mM HEPES, 10% sucrose, 0.1% CHAPS, 1 mM DTT, pH 7.0). Hydrolysis was monitored spectrophotometrically (400 nm) at 25°C.

Quantification of cell death. Percent viability was determined by morphology^{29,30}. For the percent viability assay, KS1767 cells were incubated with the concentrations of SGP and control peptide indicated in the figures. Briefly, at the given time points, cell culture medium was aspirated from adherent cells. Then the cells were gently washed once with PBS at 37°C. A 20-fold dilution of the dye mixture (100 μ g/ml acridine orange and 100 μ g/ml ethidium bromide) in PBS was then gently pipetted on the cells and viewed on an inverted microscope (Nikon TE 300). The type of cell death observed was judged morphologically^{29,30}. Apoptotic cell death was confirmed by a caspase activation assay. For apoptosis, not all cells progressed through the stages of apoptosis at the same time. At the initial stages, a fraction of the cells were undergoing early apoptosis. At later stages during the time course, this initial fraction had progressed to late apoptosis and even to the necrotic-like stage associated with very late apoptosis (e.g., loss of membrane integrity in apoptotic bodies). However, these cells were joined by a new fraction undergoing early apoptosis. Thus, cells with nuclei exhibiting margination and condens-

sation of the chromatin and/or nuclear fragmentation (early/mid apoptosis–acridine orange positive) or with compromised plasma membranes (late apoptosis–ethidium bromide positive) were scored as not viable. 500 cells per time point were scored in each experiment. Percent viability was calculated relative to untreated controls.

Human tumor xenografts. MDA-MB-435 and KS1767 derived human tumor xenografts were established in 2 month-old female, nude/nude Balb/c mice (Jackson Labs, Bar Harbor, ME) by administering 10^6 tumor cells per mouse in a 200 μl volume of serum free DMEM into the mammary fat pad, as described^{29,45}. The mice were anesthetized with Avertin as described^{29,45}. SGP was administered directly into the center of the tumor mass at a concentration of 100 μM or 1 mM given slowly in 5 μl increments, for a total volume of 40 μl . 3-D measurements of tumors were taken by caliper under anesthesia, and used to calculate tumor volume^{29,45}. Animal experimentation was reviewed and approved by the Institute's IACUC Committee.

Histology. MDA-MB-435-derived breast carcinoma and KS1767-derived Kaposi's sarcoma xenografts and organs were removed, fixed in Bouin solution, embedded in paraffin for preparation of tissue sections, and stained with hematoxylin and eosin^{29,45}.

Skin toxicity. 2 month-old female nude mice (Jackson Labs, Bar Harbor ME) were anesthetized with Avertin as described^{29,45}. 10 μl of 100 μM SGP or PBS was injected into the skin. The injected areas were monitored for 2 weeks.

Amino acid abbreviations. A, Ala; C, Cys; D, Asp; E, Glu; G, Gly; K, Lys; L, Leu; N, Asn; P, pro; R, Arg; V, Val; W, Trp; and Y, Tyr.

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*** Add in any additions for SL, WA, and RP etc.**

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Figure Legends

Fig. 1. SGP representations and mechanism. *a*, The amino acid sequence of SGP. The hydrophobic leucine and alanine residues are shown in red, and the positively charged lysine residues are shown in green. The loop residues (glycine, proline, and asparagine) are shown in blue, and the tyrosine and tryptophan residues are in black. *b*, The helical wheel diagram of SGP. *c*, The putative mechanism of SGP. In the aqueous phase SGP folds into a globular protein (upper), but adopts an inverted umbrella structure that forms a pore in lipid membranes (lower).

Fig. 2. SGP disrupts mitochondrial membranes. *a*, 1 μ M SGP or 40 μ M Ca^{+2} (positive control) induced mitochondrial swelling, whereas 100 μ M of the non- α -helix forming peptide DLSLARLATARLAI did not (negative control). Mitochondrial swelling curve (optical absorbance spectrum) is shown. *b*, 10 μ M SGP activates cell-free apoptosis in a system composed of normal mitochondria and cytosolic extract, but 100 μ M negative control peptide does not. An immunoblot of caspase-3 cleavage from proform (32 kDa) to processed forms (18 and 20 kDa) demonstrates a mitochondria-dependent cell-free apoptosis. Results were reproduced in 3 independent experiments.

Fig. 3. SGP treatment of cultured tumor cells. *a*, Human Kaposi's sarcoma derived KS1767 cells treated with 10 μ M SGP underwent extremely rapid cell death within 60 sec (black bars), while those treated with 100 μ M of negative control peptide DLSLAR-LATARLAI remain unaffected (grey bars) ($p < 0.04$). *b*, KS1767 cells treated with 10 μ M SGP undergo necrosis within 60 min (black bars), while those treated with 100 μ M of negative control peptide remain unaffected after 60 min (grey bars) ($p < 0.03$). *c*, KS1767 cells treated with 3 μ M SGP undergo apoptosis over 24 hrs, while those treated with 100 μ M of negative control peptide remain unaffected after 24 hr (grey bars) ($p < 0.05$). Hoffman contrast microscopy of KS1767 cells treated with *d*, 100 μ M of negative

control peptide for 24 hrs or *e*, 3 μ M SGP for 24 hrs. The classic morphological characteristics of condensed nuclei (short arrows) and plasma membrane blebbing (long arrows) are evident. Scale bar = 250 μ m. Results were reproduced in 3 independent experiments.

Fig. 4. SGP treatment of nude mice bearing human tumor xenografts. Data are shown for human MDA-MB-435-derived breast carcinomas. Mice had tumor volumes ranging from 100 (mm^3) (a standard starting size) to 600 (mm^3) (a large starting size) at the beginning of the experiment. *a*, SGP treated tumors are smaller than control PBS treated tumors. Differences in tumor volumes at 8 weeks are shown (*t* test, $P<0.05$, for each SGP group, $n = 5$, compared with PBS, $n = 5$). Note that a total of 10 mice received SGP. *b*, Photograph of tumors after 4 weekly treatments 40 μ l/week (see methods). The volume of the PBS treated tumor is 400 (mm^3) (left), while 100 μ M SGP (middle) and 1mM SGP (right) treated tumors have flattened-out, and virtually disappeared. These three tumors began at a volumes of 100 (mm^3). *c*, Skin toxicity of SGP. Subcutaneous injection (40 μ l) of 100 μ M SGP (left injection sight, arrow), and of PBS (right injection sight, arrow) demonstrates that SGP is relatively non-toxic to normal skin. Results for *c* were reproduced in 8 independent experiments.

Fig. 5. SGP treated tumors undergo widespread cell death. Histopathological tissue sections are shown of human tumor xenografts harvested at 8 weeks after treatment initiation. Tissue sections from human MDA-MB-435-derived breast carcinoma xenografts from nude mice treated with *a*, PBS show healthy tumor tissue but with *b*, 100 μ M SGP show extensive apoptosis with many condensed nuclei evident (short arrows), and an intact extra-cellular matrix (long arrows). *c*, and *d*, Tissue sections from human KS1767-derived Kaposi's sarcoma xenografts in nude mice show a similar outcome.

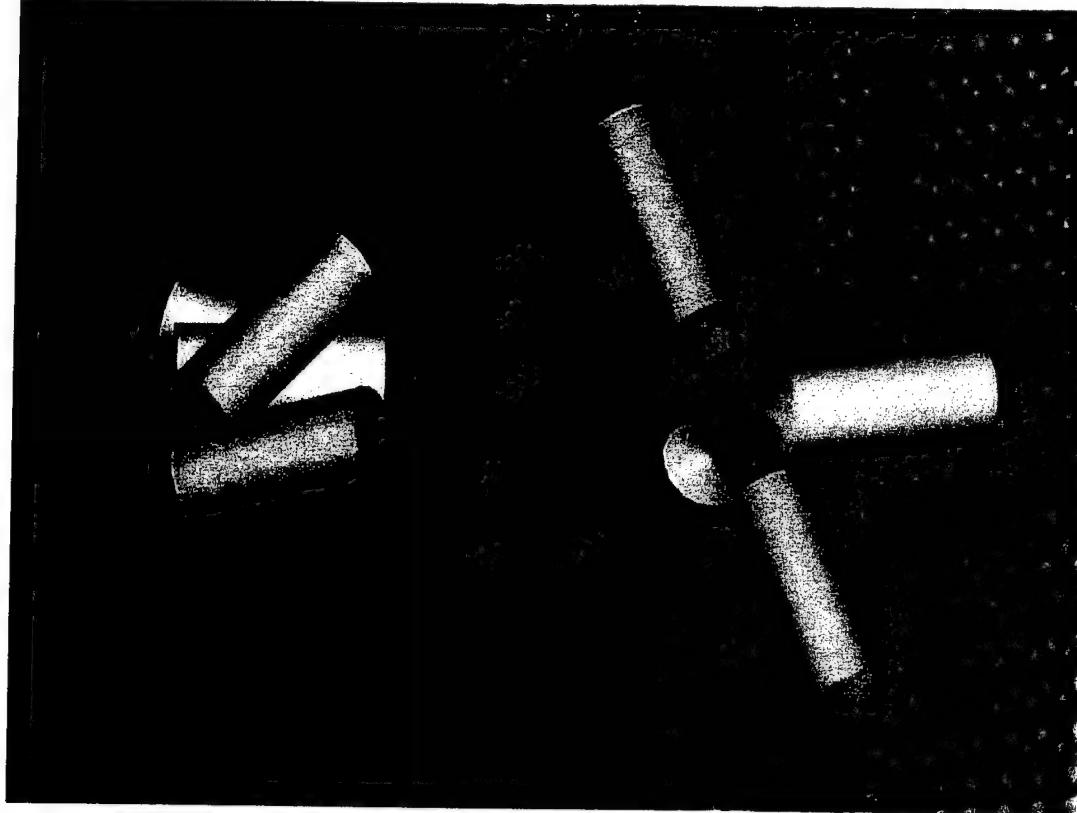
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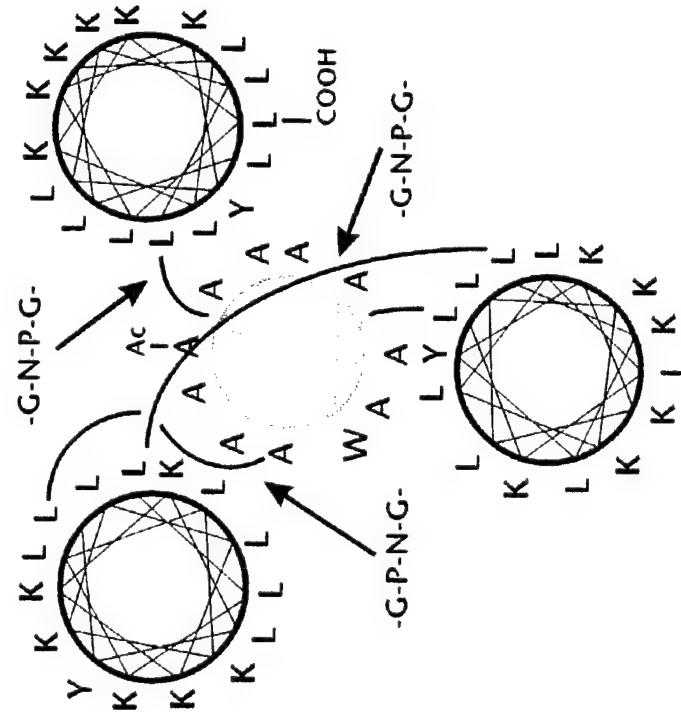
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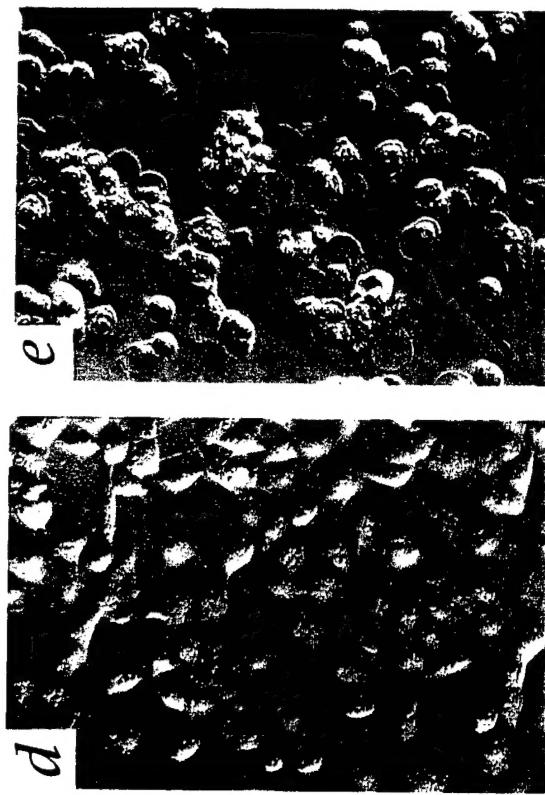
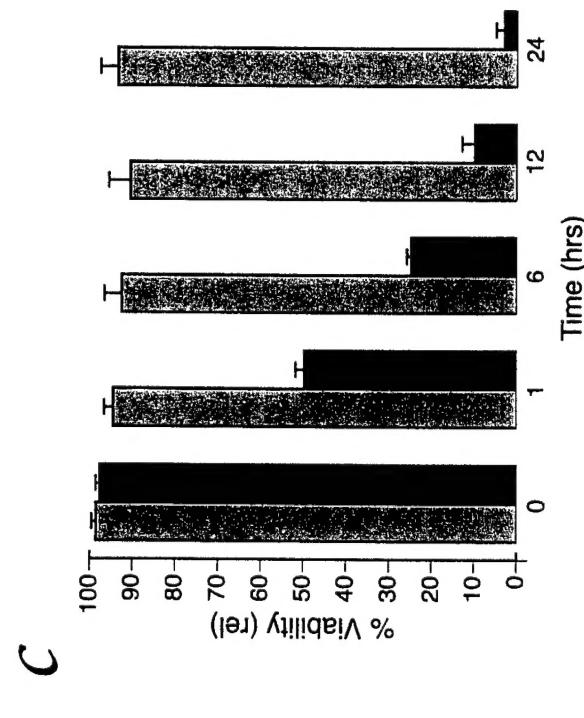
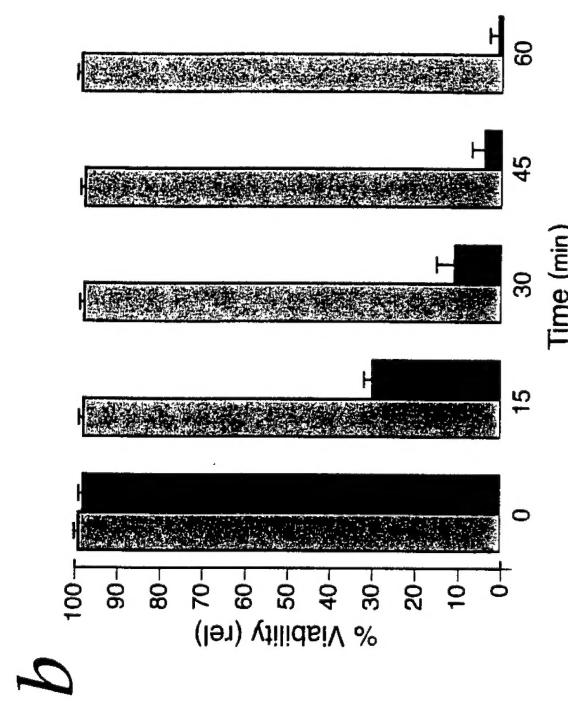
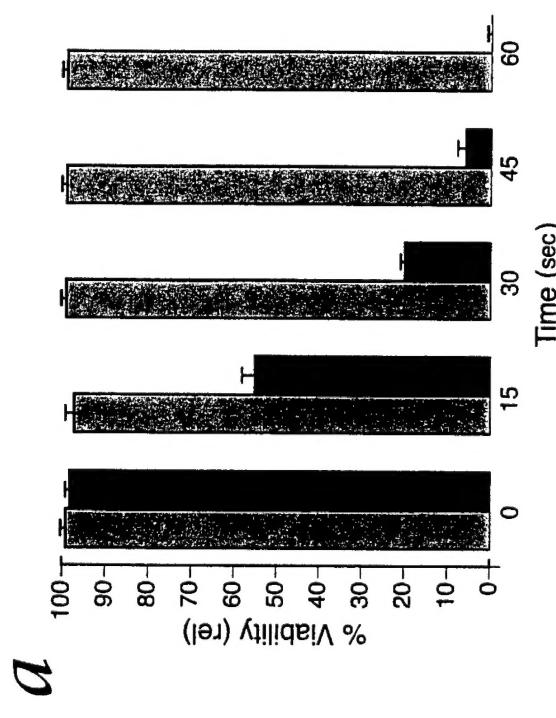
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c

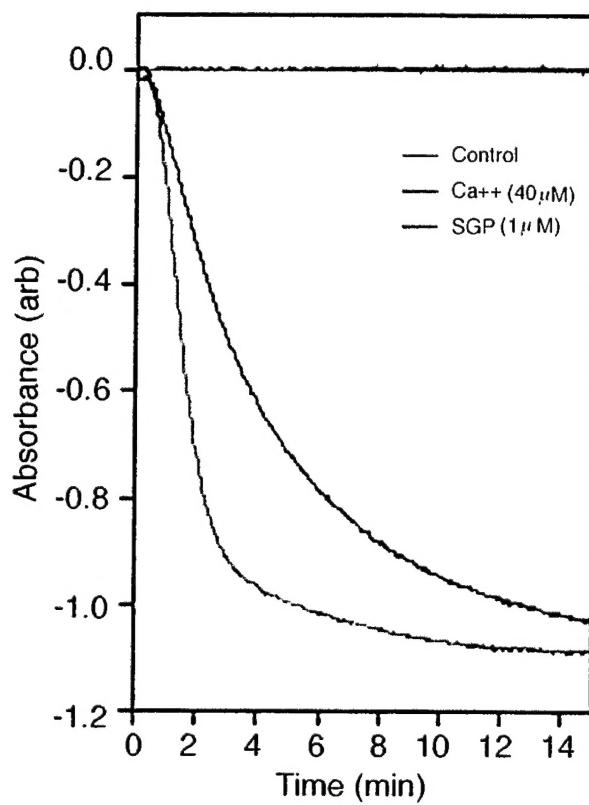


b





a



b

